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Nieland, L.; Morsett, L.M.; Broekman, M.L.D.; Breakefield, X.O.; Abels, E.R.

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## Review

## Extracellular Vesicle-Mediated Bilateral Communication between Glioblastoma and Astrocytes

Lisa Nieland,<sup>1</sup> Liza M. Morsett,<sup>2</sup> Marike L.D. Broekman,<sup>1,3,4</sup> Xandra O. Breakefield,<sup>1</sup> and Erik R. Abels<sup>1,3,\*</sup>

**Glioblastoma the most aggressive form of brain cancer, comprises a complex mixture of tumor cells and nonmalignant stromal cells, including neurons, astrocytes, microglia, infiltrating monocytes/macrophages, lymphocytes, and other cell types. All nonmalignant cells within and surrounding the tumor are affected by the presence of glioblastoma. Astrocytes use multiple modes of communication to interact with neighboring cells. Extracellular vesicle-directed intercellular communication has been found to be an important component of signaling between astrocytes and glioblastoma in tumor progression. In this review, we focus on recent findings on extracellular vesicle-mediated bilateral crosstalk, between glioblastoma cells and astrocytes, highlighting the protumor and antitumor roles of astrocytes in glioblastoma development.**

### Astrocytes within the Tumor Microenvironment

The focus of neuro-oncological research has expanded to include the cellular microenvironment of the tumor [1–3]. Hence, it is now acknowledged that tumor cells are part of a dynamic cellular network, rather than being completely independent, with this network contributing to the aggressive nature of the tumor. Glioblastoma the most malignant form of brain cancer, is characterized by its diffuse growth, heterogeneous genetic and phenotypic composition, and complex mixture of tumor cells and nonmalignant cells [3]. In this review, different grades of glioma (see Glossary) will be referred to as glioblastoma *in vivo*, since in most studies, the level of malignancy was not addressed, and the term glioma will be used for most cell culture studies according to the authors' designation. The heterogeneity of glioblastoma occurs at multiple levels, which makes this brain tumor challenging to study and currently incurable [4]. First, intertumor heterogeneity is reflected by different subtypes of tumors, which are typically classified into proneural, classical, and mesenchymal, based on transcriptional expression profiles [5,6]. These subtypes dynamically change over time, alter their induced expression profile upon therapy, and can coexist in different regions of the same tumor [7]. A single-cell sequencing study has characterized the heterogeneity of the cell states of a tumor in detail, showing that these are influenced by the **tumor microenvironment** [8].

The second layer of heterogeneity is the unique tumor microenvironment of the tumor. The cell population within the tumor as well as the tumor surroundings consists of a variety of glia subtypes, including astrocytes, oligodendrocytes, ependymal cells, and microglia, as well as infiltrating adaptive and innate immune cells, such as lymphocytes and myeloid-derived monocytes/macrophages [3]. Among these cell types, astrocytes are the most abundant. Overall, astrocytes make up about half of the total brain cell population and have many roles in both healthy and diseased brain [9]. Their functions are broad, spanning many aspects of brain (patho)physiology, and while addressing all of them in detail is beyond the scope of this review,

### Highlights

The ability of glioblastoma cells to recruit healthy brain cells into protumor cells has gained interest. This affects all cell types, including astrocytes, the homeostatic cells of the brain, which are designed to protect the brain, but may be coerced into supporting gliomas.

Astrocytes act to isolate noninjured tissue from damaged brain areas, and accordingly, migrate towards the tumor border as a possible defense mechanism. However, there is evidence that glioblastoma can transform astrocytes into an active state, defined by increased levels of glial fibrillary acidic protein (GFAP) and protumor factors.

Astrocytes maintain close interactions with tumor cells, creating complex communication networks. Extracellular vesicle-mediated crosstalk has an important role in tumor progression. Further understanding of these mechanisms may open perspectives for future therapy.

<sup>1</sup>Departments of Neurology and Radiology, Massachusetts General Hospital, and NeuroDiscovery Center, Harvard Medical School, Boston, MA, 02129, USA

<sup>2</sup>Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital and Harvard Medical School, Boston, MA, 02129, USA

<sup>3</sup>Department of Neurosurgery, Leiden University Medical Center, 2300 RC, Leiden, The Netherlands

<sup>4</sup>Department of Neurosurgery, Haaglanden Medical Center, 2512 VA, The Hague, The Netherlands

\*Correspondence: [eabels@mgh.harvard.edu](mailto:eabels@mgh.harvard.edu) (E.R. Abels).



they have been summarized in numerous reports [10–12]. There is a range of astrocyte phenotypes, as they are highly variable in morphology and function, but they can broadly be defined as ‘homeostatic cells’ because one of their main functions is the preservation of homeostasis in the healthy brain [12].

Astrocytes have an enormous impact on healthy and diseased brain functioning, and their critical role in glioblastoma progression has been increasingly recognized [11]. Therefore, it is not surprising that they maintain complex interactions with these tumor cells. Indeed, astrocytes actively interact with tumor cells, and utilize a variety of communication strategies including direct cell–cell contact through gap junctions, ion channels, microtubules, and tunneling nanotubes [13,14], and indirectly through the secretion of molecules, the release of **gliotransmitters**, and **extracellular vesicles (EVs)** [13]. These interactions influence the growth, migration, survival, and treatment resistance of brain tumors [13–16]. Here we will focus on recent insights into EV-directed communication between astrocytes and tumor cells, highlighting its contribution to glioblastoma development.

### Astrocyte Activation

To understand the role of astrocytes in glioblastoma development, it is important to discuss the process of astrocyte activation (i.e., astrogliosis). Astrogliosis is a defense mechanism of the CNS to minimize and repair initial damage from brain injuries, with the main purpose to isolate noninjured tissue from damaged brain areas; it is characterized by specific molecular, cellular, and functional alterations in glial cells, resulting in so called **reactive astrocytes** [17]. This transformation (Figure 1), in which astrocytes are activated in response to threats, such as trauma, infections, inflammation, and tumors, is characterized by increased expression of glial fibrillary acidic protein (GFAP), nestin, and vimentin [18], as well as markedly enhanced expression of the gap junction protein Connexin 43 (Cx43) [19–21]. On top of increased GFAP levels, severe brain injuries can also lead to phenotypic changes within astrocytes, characterized by cell body hypertrophy associated with the diffuse extension of distant processes [22]. These phenotypic changes have been described in mouse models extensively [22], but can also be found when studying the interactions between astrocytes and glioma cells using co-cultures (Box 1). Astrocytes are triggered into an activated state induced during glioblastoma development, and are transformed into reactive astrocytes as the tumor progresses. This was shown using transgenic mice expressing luciferase regulated by the GFAP promoter. Here, astrogliosis has been found to peak 3 days after intracranial injection of glioblastoma cells in xenograft mouse models [23]. In the latter case, astrocytes migrate directly towards the tumor border where they transform into reactive astrocytes, facilitating tumor progression, proliferation, and migration through multiple relatively well-understood signaling pathways, such as the nuclear factor kappa B (NF- $\kappa$ B) and transforming growth factor- $\beta$  (TGF- $\beta$ ) pathways [24,25]. Although this process is normally intended to protect healthy brain tissue, these signaling pathways appear to be co-opted by glioblastoma cells to change the astrocytes towards a more tumor-supportive phenotype [26]. Examples of their tumor-supportive role can be found in *in vitro* studies, where injury results in an astrocyte reactive phenotype, together with subsequent transcriptional and secretome changes associated with increased tumor cell proliferation and migration. Astrocytes activated by glioblastoma produce tumor-promoting factors, such as TGF- $\beta$ , thereby enhancing glioblastoma cell invasion [27]. In addition, signal transducer and activator of transcription 3 (STAT-3), is expressed in reactive astrocytes surrounding metastatic brain lesions [28]. Expression of this gene is also elevated in a subpopulation of reactive astrocytes, associated with metastases of peripheral tumors to the brain, and correlates with reduced patient survival time [28]. Most importantly, blocking STAT-3 had an effective antitumor effect, indicating that targeting reactive astrocytes could offer a possible therapeutic strategy against

### Glossary

#### Extracellular vesicles (EVs):

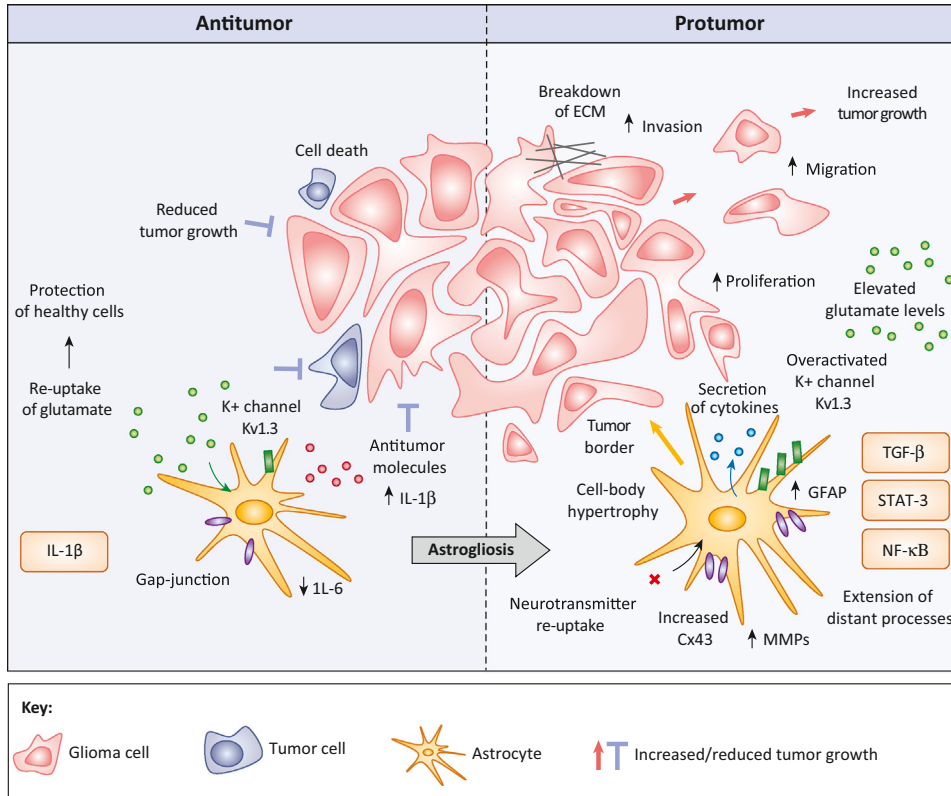
nanosized, membrane bound particles with a lipid bilayer able to transport functional cargo. Most, if not all cell-types, release EVs, which carry a heterogeneous cargo consisting of proteins, DNA, RNA, lipids, metabolites, and even organelles derived from the parent cell, and are known to have an important role in intercellular communication. EVs hold promise as diagnostic biomarkers, therapeutic targets, and therapeutic vehicles.

**Glioma:** a general term to describe the malignant tumors originated from the glia and stem cells in the brain. Glioma defines a broad category of brain and spinal cord tumors, which are named based on the specific type of brain cells affected. Gliomas are categorized in four grades according to the level of aggressiveness, and each has different types of cells present and different treatment strategies. Glioblastoma, the most common form of primary brain tumors, is a grade IV glioma, which is the most aggressive form and has three major genetic subtypes.

**Gliotransmitters:** chemicals released by all glial cell-types. Glia-derived transmitters released by astrocytes include glutamate, D-serine, and ATP, which act on neurons to regulate synaptic neurotransmission and neuronal plasticity. It is still unknown whether there is a difference in gliotransmitters between different types of astrocytes, or how they affect glioma growth.

**Reactive astrocytes:** astrocytes which have undergone astrogliosis – a process defined by proliferation and morphological alterations, such as hypertrophy, enhanced synthesis of neurotrophins and inflammatory mediators, and increased levels of GFAP. The transformation of reactive astrocytes is a widely observed phenomena and functions as a defense mechanism to minimize destruction of brain tissue after CNS injuries, including tumor development.

**Tumor microenvironment:** the area within and around the tumor, including the inclusive and surrounding blood vessels, ECM, and a mixture of stromal cell types, secreted factors, signaling molecules, and EVs. The tumor and its inclusive environment are in close contact and dynamically interactive, which affects tumor development and



progression, and even influences the therapeutic responses and resistance.

#### Trends in Neurosciences

**Figure 1. Astrocyte Activation upon Tumor Development.** Antitumor effects of astrocytes (left panel): In the early phase of tumor progression, astrocytes (orange) act to protect healthy cells from glioma cells (pink). Astrocytes express IL-1 $\beta$  and uptake neurotransmitters, such as glutamate, which protects neurons and leads to reduced tumor migration and growth, as well as increased tumor cell apoptosis. Protumor effects of astrocytes (right panel): As the tumor develops and tumor cells move into a more advanced stage, astrocytes lose the ability to re-uptake neurotransmitters, and the expression of the voltage-gated K<sup>+</sup> channel, Kv1.3, which regulates glutamate buffering, increases. These changes disturb the homeostatic properties of astrocytes and cause elevated levels of extracellular glutamate. Over time, astrocytes transform into reactive astrocytes, which are characterized by increased levels of GFAP and the gap-junction protein Cx43, and changes in morphology including a hypertrophic cell body and extended processes. The signaling pathways TGF- $\beta$ , NF- $\kappa$ B, and STAT3 are highly active in reactive astrocytes. Reactive astrocytes typically migrate to the tumor border, and the secretion of cytokines and growth factors, such as TGF- $\beta$  and MMPs are enhanced. Together, this results in a breakdown of the ECM, and increased invasion, migration and proliferation of the tumor cells. Abbreviations: ECM, extracellular matrix; GFAP, glial fibrillary acidic protein; IL-1 $\beta$ , interleukin 1 $\beta$ ; MMP, matrix metalloproteinase; NF- $\kappa$ B, nuclear factor kappa B; STAT3, signal transducer and activator of transcription 3; TGF- $\beta$ , transforming growth factor- $\beta$ .

CNS tumors [28]. Activation of astrocytes can also be caused by treatment of tumors. Upon tumor resection, as demonstrated in a murine model, the brain injury causes reactive astrocytes to promote growth and invasion of the recurrent glioma [29]. This demonstrates that defining the stimuli and mechanisms of astrocyte activation, can yield insights into potential therapeutic opportunities targeting this tumor-supportive process.

The protumor versus antitumor capacity of reactive astrocytes is a dynamic process. Interestingly, immunohistochemistry analyses of seven postmortem glioblastoma cases indicated that reactive astrocytes at the tumor edge do not express interleukin (IL)-6, important for tumor growth, but do express IL-1 $\beta$ , an immune suppressive cytokine. This may indicate that astrocytes at the tumor border are conflicted in their response. Early in tumor formation they may tend towards

### Box 1. Co-culturing Astrocytes With Glioma Cells to Study Cell–Cell Interactions

It has been challenging to study the communication between brain tumors and their microenvironment due to the paucity of adequate models. One of the first co-culture models to study glioblastoma and astrocyte interactions was reported in 1996 [79]. In co-cultures, cells were grown with some degree of contact between them, allowing multiple cell types, such as astrocytes and/or microglia, to be cultured together with tumor cells to examine the effect of one cell type on the other [80]. Co-culturing approaches may more closely represent the complex network of CNS and tumor cells rather than single-cell cultures, thus allowing the study of physiological release-uptake dynamics, and enabling the assessment of multiple mediators between the different cell types [60]. Studying the tumor microenvironment interactions is largely hampered by the difficulty of co-culturing glioma cells with primary glia, as they have distinct culture conditions. Yet, multiple co-culture assays are currently used to address cell-to-cell communication including: (i) Transwell plates [81]; (ii) microfluidic platforms [82], or solid support, such as 3D scaffolds [83]; (iii) hydrogels [84]; and (iv) microarrays [85]. These methods are used to study aspects of intracellular cross-talk, such as metabolic interactions, vessel formation, the role of glioma stem cell-like cells, and the impact of astrocytes on the response of glioma cells to different therapeutic agents [60,86]. Of note, co-culturing may shed light on nondirect cellular interactions, including EV transfer, which do not require adjacent colocalization in the tumor microenvironment. Intriguingly, patient-derived glioblastoma organoids might provide an even better model to study intercellular communication, since organoids closer recapitulate the heterogeneity of the parent tumor [87].

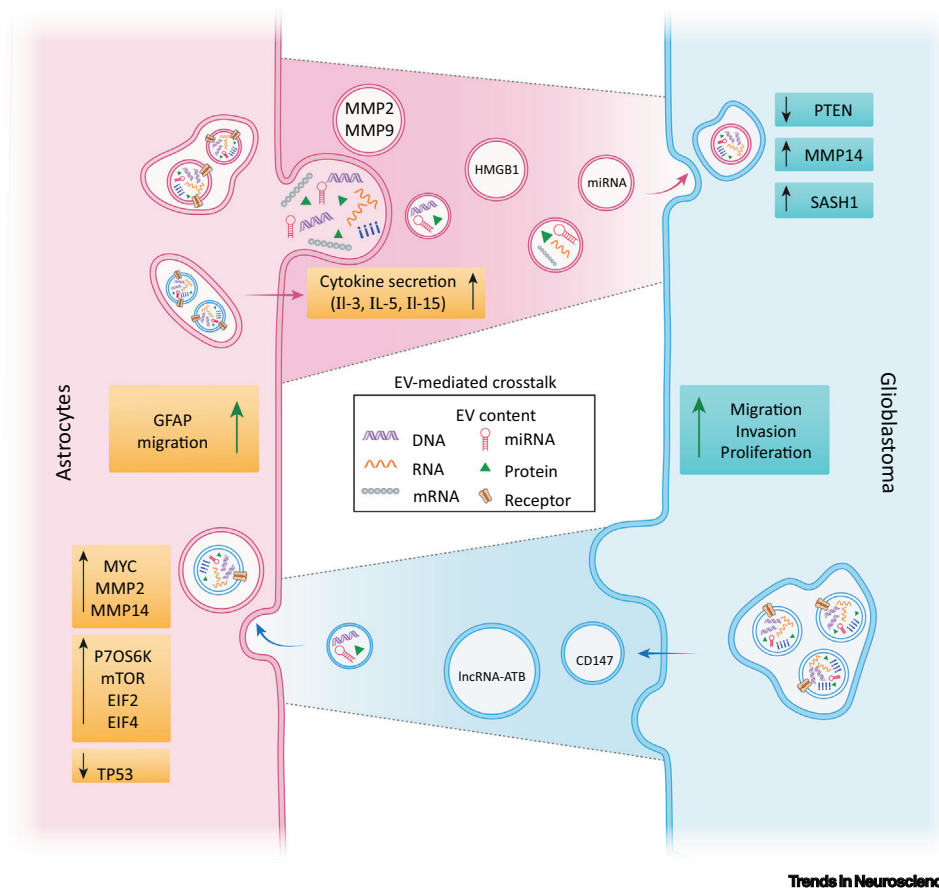
protecting healthy cells from the damage caused by the tumor [30]. The protective role of astrocytes in the context of glioma, has been shown in studies focusing on the ability of astrocytes to re-uptake neurotransmitters, such as glutamate. Glutamate is an excitatory transmitter that can cause cell toxicity when overexpressed [31]. In a healthy brain, glutamate is released from neurons and predominantly taken up by astrocytes to maintain balanced levels of glutamate [31]. In early-stage glioma development, a border of astrocytes is created around the tumor. When mixing glioma cells with astrocytes in culture it was found that large numbers of astrocytes are able to completely eliminate excessive glutamate levels, thus preventing neuronal cell death, while at the same time limiting glioma expansion, through reduction of the stimulatory role of glutamate on glioma growth. However, when glutamate is deaminated by glutaminase, ammonia is released, partly by glioma cells, which induces astrocyte swelling and dysfunction of glutamate uptake. This means, in advanced stages of glioma, astrocytes are no longer able to maintain glutamate homeostasis, as high levels of ammonia damaged the astrocytic glutamate uptake, resulting in neuronal injury providing space for tumor growth, which is beneficial for glioma progression [32]. Interestingly, the voltage-gated  $K^+$  channel, Kv1.3, regulates glutamate buffering within astrocytes. In the context of glioma, the Kv1.3 channel has increased activity which disturbs the homeostatic properties of astrocytes. The inhibition of Kv1.3 through a specific inhibitor 5-(4-phenoxybutoxy) psoralen (PAP-1), increased glutamate uptake by astrocytes, thus reducing glioma-induced neurotoxicity [33]. These findings suggest that astrocytes have a role in neuronal protection and antitumorigenic properties at an early stage in glioma development, however over time under the influence of glioma cells, astrocytes lose the ability to buffer glutamate and their protective effect on neurons. We hypothesize that, as the tumor develops, astrocytes lose their protective role and change towards a protumorigenic phenotype (Figure 1). Together, recent studies highlight how astrocytes respond to brain tumor development, which is achieved through multiple modes of communication. Here, the focus is on the EV-mediated crosstalk between astrocytes and glioblastoma (Figure 2, Key Figure).

### EV-Mediated Communication between Astrocytes and Glioblastoma

Several studies have shown that EVs, signaling vehicles with multifaceted functions in neuronal and glial crosstalk, have a crucial role in the communication among cell types within glioblastoma and its microenvironment [34,35]. EVs are membrane-enclosed nanospheres that contain proteins, lipids, and nucleic acids, such as DNA, mRNA, and noncoding RNAs, and are released by most cells [36,37]. EV-mediated communication is unique compared with other communication pathways, since it allows the delivery of combinatorial vesicle-enclosed cargo, which is not

## Key Figure

## Schematic Illustration of Bidirectional EV-Mediated Communication between Astrocytes and Glioblastoma Cells



**Figure 2.** EVs can transport different types of cargo, including DNA, RNA, receptors, and other proteins. Both astrocytes and glioblastoma cells shed EVs, carrying various cargoes as illustrated in the schematic. Moreover, EVs that originate from glioblastoma cells contain CD147, which increases MMP expression in astrocytes, and in turn contributes to the breakdown of the ECM, leading to enhanced invasion of glioblastoma cells. *Gfap* mRNA is upregulated in astrocytes, indicating a conversion to reactive astrocytes in the context of glioblastoma. Moreover, the MYC signaling pathway, an oncogene, is activated in astrocytes, whereas TP53, a tumor suppressor gene is downregulated upon glioblastoma-derived EV uptake, suggesting that astrocytes transform to tumor-like cells. Both mTOR and p70S6K signaling pathways, critical for translational regulation, and initiation and control of protein synthesis, are upregulated in astrocytes as a result of exposure to glioblastoma-derived EVs. Concurrently, EVs shed by astrocytes contain multiple PTEN targeting miRNAs, including miR-19b and miR-20a, leading to a decrease in PTEN expression in the tumor cells, which enhances tumor cell proliferation. In addition, astrocytes induce an upregulation of MMP14 in glioblastoma cells, which promotes ECM degradation and tumor cell migration. Taken together, glioblastoma cells are able to alter astrocytes by the release of EVs, which change the phenotype of astrocytes to become supportive of tumor progression. The ‘transformed’ astrocytes shed EVs supporting glioblastoma cells instead of defending the CNS. Abbreviations: ECM, extracellular matrix; EV, extracellular vesicle; GFAP, glial fibrillary acidic protein; IL, interleukin; IncRNA-ATB, long noncoding RNA activated by TGF- $\beta$ ; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin.

soluble, and is protected from degradation, with access not only to the neighboring tumor cells, but also to distant sites [38–40]. A number of studies have demonstrated that the tumor micro-environment, particularly the stromal cells in the tumor, contributes to the malignant behavior of

tumor cells [3,41,42]. Almost all normal cells within and surrounding the tumor are affected by the glioblastoma, including astrocytes [1].

#### Effects of Glioblastoma-Derived EVs on Astrocytes

Importantly, glioblastoma-derived EVs can be taken up by different cells in the tumor environment, such as endothelial cells, microglia, monocytes [43–45], and astrocytes [46]. EVs represent one of the mechanisms by which cancer cells modulate the tumor microenvironment to stimulate their growth, invasion, neo-angiogenesis, oncogenic transformation, and suppression of the immune response [47,48]. Evidence suggests that the content of glioblastoma-derived EVs, at least to a certain extent, mirrors the phenotypic and genotypic signature of the respective glioblastoma cells. This supports the use of EV-associated biomarkers, such as CD44, a multifunctional plasma membrane protein, with an important predictive value for glioblastoma subtyping [49,50], and detection of tumor driver mutations, such as epidermal growth factor receptor (EGFR)vIII [51,52].

What is the functional effect of the uptake of glioblastoma-derived EV by astrocytes? One study found that uptake of EVs derived from surgically resected primary glioblastoma tumors, drove primary human astrocytes towards a tumor-enhancing phenotype in culture [46]. Glioblastoma-derived EVs taken up by primary human astrocytes *in vitro* increased their migratory capabilities, activated oncogenic signaling pathways, including epidermal growth factor (EGF), receptor-type tyrosine-protein kinase (FLT), IL-22, and mitogen activated protein kinase (MAPK), and converted them into reactive astrocytes defined by increased *Gfap* levels.

From a neurodevelopmental perspective, the increase in GFAP levels raises the question whether these astrocytes are part of the tumor stem cell evolution. Interestingly, abnormal development of glial progenitors, including astrocyte precursor cells, appear to contribute to tumorigenesis [53], and gliomas consist of a substantial proportion of glial progenitor-like cells [54]. In addition, it was revealed that distinct glial progenitors present in brain development, are similar to the progenitors present during tumorigenesis [55,56].

Another observation showing the dynamics between astrocyte phenotype and tumors, is found in the notion that astrocytes undergo changes based on their location in the healthy and pathological brain [57–59]. This all indicates that astrocyte progenitors can potentially transform into glioma cells, which can also be driven by glioma-derived EVs. To start, it was confirmed that these two cells types communicate by showing that extracellular RNA was transferred from glioma stem cells to astrocytes through EVs [60]. Interestingly, glioblastoma-derived EVs facilitated neoplastic growth of astrocytes, but not normal human or mouse astrocytes, by reprogramming glycolysis and oxidative phosphorylation through EV-mediated transfer of miRNAs, inducing proliferation, self-renewal, and colony formation of activated astrocytes, and enhanced malignant growth of astrocytes in a mouse allograft model [61]. *In vitro* studies have also shown that transformed astrocytes have an increased ability to take up EVs. In detail, the spontaneous cellular transformation of human astrocytes during serial culturing resulted in a higher level of EV uptake [62]. The implications of this observation, and whether this also occurs during the activation of protumorigenic astrogliosis, is still to be examined. However, EVs released by patient-derived glioblastoma stem cells do stimulate astrocytes to acquire a transformed-like phenotype through inhibition of TP53, an important tumor suppressor gene [63], in combination with activation of MYC, a proto-oncogenic signaling molecule important in the regulation of cell proliferation [64]. Thus, changes in these signaling pathways in normal astrocytes exposed to glioblastoma-derived EVs, reveals mechanisms by which glioblastomas manipulate astrocytes to create a tumorigenic microenvironment [65].

In addition, EVs isolated from genetically unstable, oncogene-driven glioma cells, can trigger the formation of micronuclei in neighboring cells, which are associated with genotoxicity and chromosomal instability. Micronuclei were found in both endothelial cells and hTERT-immortalized human astrocytes, exposed to glioma-derived EVs, demonstrating their impact on chromosomal stability in recipient cells [66]. Extensive proteomic analyses have been performed on astrocytes treated with EVs derived from glioblastoma stem-like cells, compared with untreated astrocytes. Here, EIF2, eIF4, p70S6K, and mammalian target of rapamycin (mTOR) signaling pathways, critical for translational regulation, and initiation and control of protein synthesis, were shown to be significantly enhanced, stimulating proteome changes and mediating a transformed phenotype in astrocytes treated with glioblastoma-derived EVs [65]. Taken together, glioblastoma-derived EVs can morph astrocytes into tumor-like cells.

More indications of the functional consequences of EVs were found when astrocytes were incubated with glioblastoma-derived EVs and the medium collected from these cells, with medium collected from astrocytes, not exposed to glioblastoma-derived EVs, serving as a control. Interestingly, glioblastoma cells incubated, in turn, with the 'EV-treated astrocyte growth medium' proliferated at a significantly faster rate than the same cells treated with 'control astrocyte growth medium', as measured by a NAD(P)H-dependent dehydrogenase-based assay evaluating the metabolic activity of cells [46]. In parallel, by using a rat glioma cell line, treatment of astrocytes with tumor cell-derived EVs increased the rate of astrocyte proliferation and elevated the mRNA expression levels of *Gfap* [67]. Several pieces of evidence showed that this is consistent with the ability of glioblastoma cells to convert astrocytes into reactive astrocytes, as measured by increased GFAP expression, mediated at least in part via EVs [68]. One of the mechanisms through which astrocytes can be activated, is through the EV-mediated transfer of the glioma-derived long noncoding RNA (lncRNA), activated by TGF $\beta$ , (lncRNA-ATB) [68]. Upon uptake, this glioma-derived lncRNA downregulates miR-204-3p in astrocytes, leading to an increase in miR-204-3p mRNA target *Gfap*. This lncRNA-ATB, related to the activation of astrocytes, is also associated with increased glioma cell invasion [68]. Importantly, the involvement of a specific lncRNA in increased glioma cell invasion, provides a promising opportunity to target this lncRNA for therapeutic purposes.

Why would the activation of astrocytes be impactful in glioblastoma development? One possible explanation is because it is accompanied by the increased ability to breakdown the extracellular matrix (ECM) through elevated matrix metalloproteinase (MMP) levels, which is important for tumor growth and invasion [68]. The secretion and overexpression of MMPs, including MMP2, MMP9, and MMP14, by astrocytes exposed to glioblastoma-derived EVs, contributes to protease-based destruction of the ECM to facilitate glioblastoma cell invasion [46,67]. As discussed earlier, treatment of tumors can have an adverse effect through astrocytes, a process which can be mediated by EVs [28]. In response to IR, glioblastoma cells increase the levels of CD147 shed in EVs. This protein, involved in regulation of MMPs, enhances MMP9 release from astrocytes exposed to these EVs by increasing JNK signaling, a process that was blocked by knocking out CD147 in glioma cells [69]. Since MMPs break down ECM, this result supports a tumor microenvironment-mediated role of CD147 in glioblastoma invasiveness, and reveals a prominent role for IR, used in treatment for glioblastoma, in enhancing this effect [69]. In addition, astrocytes exposed to glioblastoma-derived EVs were tested in an 'invadopodia' assay to determine the effects on astrocyte migration and ECM degradation [65]. Invadopodia are specialized actin-based dynamic cell membrane protrusions that degrade the ECM to facilitate cell migration. Interestingly, the gelatin degradation ability of astrocytes was significantly enhanced after treatment with glioblastoma-derived EVs relative to untreated cells, supporting the breakdown of the ECM in assisting glioblastoma cell invasion

[65]. These data suggest that through the secretion of different MMPs, astrocytes facilitate in the remodeling of the tumor surroundings.

To further support growth and invasion of the tumor, astrocytes can also create an immune favorable environment for glioma cells. Human astrocytes treated with glioblastoma-derived EVs showed increased expression of interferon gamma (IFN- $\gamma$ ), IL-12, IL-1A, IL-8, and IL-1 $\beta$ , chemokine CXCL10, and factor C5, compared with astrocytes exposed to EVs derived from normal epithelial cells [46]. Several of these cytokines, such as colony-stimulating factor (CSF)2 and 3, and IL-4, -10, and -13, secreted by astrocytes upon treatment with glioblastoma-derived EVs, are thought to regulate T cells and promote immune suppression [46,63]. In addition, uptake of glioblastoma-derived EVs upregulated the release of certain cytokines from astrocytes, such as the proinflammatory cytokine IFN $\gamma$  and various interleukins (IL-1A, IL-1 $\beta$ , IL-8, and IL-12), implicating an autocrine stimulation of astrocytes to maintain the inflammatory milieu in a brain tumor setting [46]. Together, the proinflammatory cytokine environment of astrocytes treated with glioblastoma-derived EVs could potentially promote immune suppression, which is highly prevalent in glioblastoma patients.

While numerous studies have examined the EV-mediated communication in culture, the first indication (to our knowledge) of the communication between glioma cells and astrocytes *in vivo*, was achieved using a transgenic mouse model [70]. In this model, astrocytes express the fluorescent marker tdTomato under a tamoxifen-inducible Cre-regulated GFAP promoter, and GFP-expressing glioma cells were transplanted into the brains of these mice. The interaction between astrocytes and glioma was monitored over 10 days post injection using intravital imaging. Here, the first indication of glioma-derived EV transfer, was detection of GFP-positive astrocytes. Combining the Cre/LoxP system with longitudinal time-lapse live imaging, GFP/Cre-expressing glioma cells were found to transfer Cre to stromal cells as measured by tdTomato expression resulting from Cre recombinase activity. The majority of the tdTomato-positive cells were identified as astrocytes by GFAP staining. Interestingly, after EV purification by ultracentrifugation, both full length Cre and GFP mRNAs were present in EVs derived from glioma cells, supporting their ability to transport Cre and GFP mRNA, and proteins derived from the tumor to astrocytes. To confirm that glioma-derived EV could transfer Cre to astrocytes, isolated EVs from GFP/Cre expressing glioma cells were injected into one hemisphere of C57BL/6J mouse brains. EV-mediated Cre transfer and subsequent recombination was detected by the presence of tdTomato-positive, GFAP positive astrocytes, eight days post injection.

There are several outstanding questions related to effects of therapy on EV-mediated communication between astrocytes and glioma, and whether therapeutic directions should be tethered towards this interaction. The first indication that EV-mediated communication between astrocytes and glioma cells could be changed under the influence of therapy, was found in the observation that chemotherapy (i.e., temozolomide) alters the composition of EVs shed by glioma cells [71]. Whether this results in a protumor or antitumorigenic switch of astrocytes after EV uptake needs to be studied.

Taken together, these results support the profound effect of glioblastoma-derived EVs on neighboring astrocytes, which contributes to alterations in the tumor microenvironment in ways which are beneficial to tumor progression. Future studies should focus on the effects of standard therapy on the uptake of EVs and subsequent phenotypical changes of the astrocytes.

#### Effects of Astrocyte-Derived EVs on Glioblastoma

What effect do EVs secreted by astrocytes have on glioblastoma development? A growing body of evidence indicates that astrocytes, like many other cell types, use EVs to transfer information.

The shedding of EVs from astrocytes was shown to be partly dependent on the activation of acidic sphingomyelinase [72]. Astrocytes are able to secrete both small and large EVs (ranging from 350 nm to 6.5  $\mu$ m as determined by electron microscopy) from the cell-surface, with the latter containing lipid droplets, ATP, and mitochondria, which possibly could be defined as apoptotic bodies [73]. Like most different cell types, astrocytes shed EVs with a heterogeneous composition in size and cargo, that mediate both physiological and pathological functions. EVs are able to transfer pathogenic proteins, suggesting that EVs may contribute to particular pathologies [74].

It is thought that astrocytes initially act as a defensive mechanism against glioblastoma cells, since the presence of the tumor activates them, presumably as a protective shield for the surrounding neurons [75]. As discussed earlier, in response to glioblastoma infiltration, astrocytes undergo a series of structural and functional changes, first changing into reactive astrocytes associated with repair of the affected tissue. However, in the case of glioblastoma development, this process seems to be quickly diverted to support tumorigenesis and further contributes to tumor growth and invasion, including resistance to treatment (Figure 1) [26]. In co-culture models of astrocytes and brain metastatic tumor cells, EVs shed by astrocytes were found to decrease levels of the tumor suppressor gene, PTEN, in tumor cells. Here, PTEN-targeting miRNAs, including miR-17, miR-19a, miR-19b, miR-20a, and miR-92, which are transported via EVs from astrocytes to brain metastatic tumor cells, are involved in PTEN downregulation. This study showed that both human and mouse tumor cells have decreased PTEN expression, after dissemination to the brain, but not to other organs, indicating that PTEN is a key factor in priming the brain tumor microenvironment and critical for tumor growth [76]. Although recent studies have reported both protumor and antitumor roles for astrocytes in glioblastoma progression, the balance of evidence seems to point toward a protumorigenic role for astrogliosis as the tumor progresses, with the release of growth factors and matrix remodeling enzymes. Although EV-mediated communication may play an important role in this transformation, further studies regarding astrocyte-derived EVs are necessary, to establish a more complete picture of the complex tumor cell-astrocyte networks [77].

### Concluding Remarks

Recently, the crosstalk between glioblastoma cells and astrocytes through the transfer of EV cargo has become an area of interest. Here, the balance of evidence seems to point toward a protumorigenic role for astrocytes in later stages of glioma development, promoting tumor growth, migration, and invasiveness. While the EV community has sought to provide consistent guidelines for how to isolate and setup EV-oriented experiments [78], the different findings discussed in this review, on the role of EVs in astrocyte–glioblastoma communication, used a variety of techniques (Table 1). Of note, the EV incubation times show a clear distinction between acute and chronic exposure, potentially affecting the reprogramming of cells present in the tumor microenvironment differently. For future research it will be important to adhere to the aforementioned guidelines, to be able to qualitatively compare different studies. That said, one can conclude that glioblastoma-derived EVs participate in astrocyte activation, in support of the migration and proliferation of glioma cells *in vitro* [46,65], and glioblastomas *in vivo* [70]. Unraveling the biology of EV exchange between astrocytes and tumor cells, may provide important therapeutic options to impede the transfer of genetic material and other cargo, and wrest control of the tumor microenvironment from glioblastoma cells. However, there are many unresolved questions regarding communication between glioblastoma cells and astrocytes via EVs (see Outstanding Questions). Now that more sophisticated animal models are available [70], the field can begin to clarify the complex role of astrocytes in the tumor microenvironment *in vivo*. Functional imaging techniques, such as methods visualizing calcium signaling, will be

### Outstanding Questions

To what extent do the different types of EVs secreted by glioblastoma cells influence astrocytes? Does the transition to protumorigenic astrocytes lead to increased EV uptake by astrocytes? Are there ways to suppress specific EV-mediated communication?

Astrocytes collectively form a border around the tumor edge. Is this migration due to signals transferred through EVs?

The glial scar is claimed to function as a defense mechanism of the brain to protect non-tumor cells from being invaded by glioblastoma cells. Many studies, however, have shown that these tumor-associated astrocytes become tumor supportive. This raises the question, do all astrocytes become protumorigenic over time, or are there different subtypes of astrocytes that are 'lured' into the tumor microenvironment? To what extent is this dependent on the glioma subtype?

Now that more sophisticated animal models are available, scientists can start to investigate the complex interactions between astrocytes and the tumor microenvironment. How can these results be translated to humans?

Astrocytes are influenced by the uptake of glioma-derived EVs. Can EVs loaded *ex vivo* or *in vivo* with specific compounds be used as therapeutic agents?

Table 1. EV Procedures Used in Different Studies

| Method                            |                     | Cell lines                                  |  | EV handling  |  | Refs |
|-----------------------------------|---------------------|---|--|--|--|------|
| Assay                             | EV incubation       | Glioblastoma                                | Astrocytes   | EV isolation   | EV characterization  |      |
| <i>In vitro</i><br><i>In vivo</i> | 12 and<br>24 h      | T98G<br>U-87 MG<br>U-118 MG<br>Human glioma | Human fetal glial cells<br>SVG (ATCC) <sup>a</sup> | Ultracentrifugation                                      | Nanoparticle tracking analysis (NanoSight)   | [69] |
| <i>In vitro</i>                   | 7 days              | U373P<br>U373VIII<br>Human glioma           | Normal human<br>astrocytes                         | Ultracentrifugation                                      | Transmission electron microscopy   | [66] |
| <i>In vitro</i><br><i>In vivo</i> | Not done            | 73C Mouse glioma                            | Mouse primary<br>astrocytes                        | Ultracentrifugation                                      | <ul style="list-style-type: none"> <li>• Transmission electron microscopy</li> <li>• Dynamic laser scattering technique</li> </ul>                           | [70] |
| <i>In vitro</i>                   | 24 h                | U-87 MG Human<br>glioblastoma               | Human primary<br>astrocytes                        | Ultracentrifugation<br><br>Iodixanol density<br>gradient | <ul style="list-style-type: none"> <li>• Nanoparticle tracking analysis (NanoSight)</li> <li>• Invadopodia assay Transmission Electron Microscopy</li> </ul> | [65] |
| <i>In vitro</i>                   | 4, 8, and<br>24 h   | Surgically resected<br>primary tumors       | Normal human<br>astrocytes                         | Ultracentrifugation                                      | <ul style="list-style-type: none"> <li>• Nanoparticle tracking analysis (NanoSight)</li> <li>• Exo-Check arrays (System Biosciences)</li> </ul>              | [46] |
| <i>In vitro</i>                   | 1, 3, and<br>5 days | C6 Rat glioma                               | Normal human<br>astrocytes                         | Ultracentrifugation                                      | <ul style="list-style-type: none"> <li>• Dynamic light scattering</li> <li>• Transmission electron microscopy</li> </ul>                                     | [67] |
| <i>In vitro</i><br><i>In vivo</i> | 2 and<br>5 days     | GBM8<br>GBM4<br>Human glioma                | Normal human<br>astrocytes                         | Ultracentrifugation                                      | <ul style="list-style-type: none"> <li>• Transmission electron microscopy</li> </ul>   | [61] |
| <i>In vitro</i>                   | 7 days              | U373p<br>U87<br>Human glioma                | Normal human<br>astrocytes                         | Ultracentrifugation                                      | <ul style="list-style-type: none"> <li>• Transmission electron microscopy</li> </ul>   | [62] |

<sup>a</sup>Abbreviation: ATCC, American type culture collection.

important in understanding the homeostatic properties of astrocytes, and other novel imaging approaches will allow a better understanding of how astrocytes communicate and respond within the tumor microenvironment. Not only do these techniques potentially allow visualization and quantification of EV-mediated communication, but they may yield mechanistic insights that could potentially lead to novel therapeutic approaches. Taken together, further investigation of the intercellular communication between glioblastoma cells and astrocytes within their surroundings, will bring researchers closer to understanding glioblastoma biology.

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