

## REVIEW

## Expression of P-gp in Glioblastoma: What we can Learn from Brain Development

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**Abstract:** P-Glycoprotein (P-gp) is a 170-kDa transmembrane glycoprotein that works as an efflux pump and confers multidrug resistance (MDR) in normal tissues and tumors, including nervous tissue and brain tumors. In the developing telencephalon, the endothelial expression of P-gp, and the subcellular localization of the transporter at the luminal endothelial cell (EC) plasma membrane are an early hallmark of blood-brain barrier (BBB) differentiation, and suggest a functional BBB activity that may complement the placental barrier function and the expression of P-gp at the blood-placental interface. In early fetal ages, P-gp has also been immunolocalized on radial glia cells (RGCs), located in the proliferative ventricular zone (VZ) of the dorsal telencephalon and now considered to be neural progenitor cells (NPCs). RG-like NPCs have been found in many regions of the developing brain and have been suggested to give rise to neural stem cells (NSCs) of adult subventricular (SVZ) neurogenic niches. The P-gp immunosignal, associated with RG-like NPCs during cortical histogenesis, progressively decreases in parallel with the last waves of neuroblast migrations, while 'outer' RGCs and the deriving astrocytes do not stain for the efflux transporter. These data suggest that in human glioblastoma (GBM) P-gp expressed by ECs may be a negligible component of tumor MDR. Instead, tumor perivascular astrocytes may dedifferentiate and resume a progenitor-like P-gp activity, becoming MDR cells and contribute, together with perivascular P-gp-expressing glioma stem-like cells (GSCs), to the MDR profile of GBM vessels. In conclusion, the analysis of P-gp immunolocalization during brain development may contribute to identify the multiple cellular sources in the GBM vessels that may be involved in P-gp-mediated chemoresistance and can be responsible for GBM therapy failure and tumor recurrence.

**Keywords:** P-glycoprotein, blood-brain barrier, endothelial cells, astrocytes, radial glia-like neural progenitor cells, glioma stem cells, developing brain, adult brain, glioblastoma.

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## 1. INTRODUCTION

In the central nervous system (CNS), highly specialized metabolic and efflux transporters contribute to the activities of the blood–brain barrier (BBB), a sophisticated endothelial cell (EC) device incorporated in the neurovascular unit (NVU), the brain microvessel entity formed by a number of highly specialized and hyperconnected cells. In the CNS, the BBB/NVU regulates neural tissue homeostasis and is involved in the response to neuropathological noxae. Part of the BBB activity is ensured by multidrug resistant (MDR) efflux transporters that prevent the passage of neurocytotoxic molecules within the nervous tissue. P-glycoprotein (P-gp), also known as MDR1 and ABCB1, one of the most common efflux transporters in the brain, is a member of the ATP-binding cassette (ABC) superfamily, encoded by the *ABCB1* (adenosine triphosphate-binding cassette, subfamily B, member 1) gene. P-gp is a 170-kDa transmembrane glycoprotein functioning as an energy-dependent efflux pump, involved in vectorial back-transport of a wide range of lipophilic, amphipathic xeno- and endo-biotic compounds, including potentially neurocytotoxic chemical substances and drugs [1-6]. Initially, the existence of an efflux pump with a broad range of substrates was demonstrated by biochemical and pharmacological studies in tumor cells that displayed the MDR phenomenon. Subsequently, a wide variety of normal epithelial cells, placental cells, and vascular cells have also been shown to express P-gp under physiological conditions [7, 8]. The general interest in MDR transporters *de facto* results from their double-sided functional activity, primarily protective in normal brain, while possibly adverse in CNS diseases, such as congenital enzyme deficiencies, neurodegenerative diseases, and malignant brain tumors, where they can cause the failure of therapeutic interventions. Glioblastoma (GBM) is one of the highly aggressive primary brain malignancies, characterized by the presence of intrinsic and/or acquired P-gp-mediated resistance to anticancer drugs, one of the main reasons for chemotherapy failure [9-11]. This review focuses on the less known and/or debated data on P-gp cellular and subcellular immunolocalization in both normal and tumor tissues, with the aim of contributing to advances in the understanding of tissue ‘defense/resistance’ by analyzing P-gp in human fetal telencephalon and adult cerebral cortex together with human GBM.

## 2. P-GLYCOPROTEIN IMMUNOLOCALIZATION IN HUMAN DEVELOPING BRAIN

Interesting data on the expression of P-gp during brain development have been discovered in the last two decades. In 1999 Matsuoka and colleagues [12] detected P-gp in rat brain by immunoblotting and immunohistochemical (IHC) studies, as early as postnatal day 7, showing a gradual increase in the subsequent weeks, to reach a plateau in the adult brain. In brain capillaries, P-gp was localized on microvascular ECs, while perivascular astrocytes did not express the transporter [12]. Subsequent studies

carried out on rodent brains by Western blot, mRNA, RT-PCR, and IHC analyses described the physiological regulation of P-gp during prenatal ontogeny and confirmed its developmentally regulated increase and the maximum level of expression reached in adult animals, together with a significant decrease in aging brains [13, 14]. It has been suggested that the activity of P-gp at the developing BBB complements the function of the placental barrier, where the efflux transporter is localized at the syncytiotrophoblast-blood interface [15]. In humans, Schumacher and Mollgård [16] described a detectable endothelial expression of P-gp in brain microvessels of embryos of about 30-mm crown-rump length (CRL) (gestational age 9,6/weeks+days), that changed to a strong expression with increasing fetal age. We contributed to P-gp immunolocalization in human developing brain, analyzing the efflux transporter in the dorsal aspect of the telencephalic vesicles (future neocortex and subcortical white matter), to better define, by high resolution laser confocal microscopy, its cellular and subcellular localization [15]. According to these results, P-gp is detectable on the endothelial lining of cortex microvessels at the earliest examined age, 12 weeks of gestation (wg). No differences between the cortex and the subcortical layers are observed, and its staining pattern increases and shifts from the cytoplasm to the endothelial cell membranes at 18 through 22 wg (Fig. 1A-C). In developing brain, like in adult brain (see below), any detectable P-gp staining was revealed by double immunostaining with the astroglia marker GFAP on parenchymal and perivascular astrocytes at 22 wg (Fig. 2A, B; Supplementary Fig. 1A, B). The subcellular localization of the transporter shows that P-gp is concentrated on the luminal endothelial cell membrane, although also present on the abluminal one (Fig. 2A, B). However, data on P-gp expression in developing prenatal and postnatal human CNS remain very scarce [16-18]. Recently, in a study on the heightened sensitivity of neonates, compared to older infants and adults, to the central depressive effects of opioids, a limited expression of P-gp at birth has been demonstrated, which increases postnatally and reaches adult levels at ~3–6 months of age [19]. It is conceivable that the late prenatal and the early postnatal ages could be the most sensitive to the effects of neurocytotoxic molecules, if P-gp activity at the BBB is not paralleled by the efficacy of the blood-placental barrier at term. In fact, unlike in rodent placenta, where an increase of P-gp was observed towards term [20], in human placenta the decreases of P-gp with the advancing gestational age [21], not balanced by an adequate increase of P-gp at the BBB [16], could be unable to ensure a full spectrum of protection in late prenatal and early postnatal ages. The hypothesis that the BBB can form a second line of defense during the sensitive phases of human cerebral cortex development, thus having a functional activity, is supported by the analysis of P-gp/caveolin-1 relationships in fetal brain microvessels (Fig. 3A, B). The presence of a caveolar compartment in brain microvessel endothelial cells has been revealed by biochemical procedures and immunocytochemical studies, which identified a number of caveolin subtypes in bovine and rodent

brains. *In vivo*, rat brain microvessels stain for caveolin-1 and -2, whereas astrocytes express caveolin-3, *in vivo*, and caveolin-1 and -2 *in vitro* [22, 23]. Studies carried out by immunoelectron microscopy revealed a prevalent association of caveolin-1 with the abluminal contingent of BBB-endothelial cell caveolae [24], where caveolin-1 and the mitogen-activated protein kinase (MAPK) signaling pathway mutually interact, regulating the ABCB1/P-gp-mediated efflux [25, 26]. Interestingly, co-localization of P-gp and caveolin-1 has been found in caveolae of BBB microvessels (Fig. 3A, B), indicating that this association takes place not only *in vitro* but also *in vivo*. Caveolin-1 in its phosphorylated (Tyr-14) form may exert a negative effect on P-gp activity, followed by the accumulation of P-gp-specific substrates in BBB endothelial cells [15, 27-31].

### 3. P-GLYCOPROTEIN IMMUNOLOCALIZATION IN HUMAN ADULT BRAIN

Although data on the molecular expression of P-gp by the main NVU cell components, ECs, pericytes, and perivascular astrocytes, still appear controversial, the relevance of the NVU function in brain protection from a vast and diverse array of toxicants has been confirmed by a variety of methodological approaches in different species. Numerous studies, in both rodent and human adult brain, have established that P-gp is present in microvessel ECs (Figs. 1D-F; 2C, D) [3, 15, 27, 32-34] and, within the NVU, perivascular astrocytes have also been detected to express P-gp [35-38]. However, our immunohistochemical analysis was unable to detect a P-gp signal on astrocytes, while it revealed the presence of P-gp on microvascular pericytes and smooth muscle cells (Fig. 2C, D) [15, 27]. Interestingly, immunoelectron microscopy carried out on rat brain confirmed that P-gp localizes on pericyte plasma membranes, also showing the site of a possible functional interaction at the caveolar compartment of pericytes [24, 38]. In humans, where P-gp activity acquires a great relevance, being described as three- to five-fold higher than in murine and bovine brains [39], the regulating mechanisms that involve P-gp and caveolin-1, as well as their respective cellular and subcellular distribution, need to be further explored, although observations of P-gp and caveolin-1 immunosignal by high resolution confocal microscopy have allowed a better definition of the sites of their co-localization (Fig. 3C, D).

### 4. P-GLYCOPROTEIN IMMUNOLOCALIZATION IN GBM

Glioblastoma, previously known also as glioblastoma ‘multiforme’ (GBM) due to the high variance of its tissue and cellular components and the resulting combined histopathological profiles, is the most malignant of CNS tumors, with an average incidence of 3 to 5 per 100,000 people per year in Europe [40]. It accounts for approximately 15% of all primary brain and CNS tumors, and for approximately 45% of all

gliomas. GBM hallmarks include: neural cells dedifferentiation, tumor cells heterogeneity, aberrant angiogenesis/vasculogenesis, BBB breakdown, and MDR transporters overexpression. For the treatment of GBM, surgery is the cornerstone but even in cases with macroscopically radical resection, surgery generally fails and needs to be followed by chemotherapy with temozolomide and concomitant radiotherapy, according to the established Stupp protocol [41]. However, despite improvements in the medical care of patients with GBM, more advanced surgical techniques, the introduction of second line medical treatment (nitrosureas or bevacizumab) or the emerging role of Tumor-Treating Fields (TTFs), the overall survival increased very little, from 14.6 months [42] to 19.8 months in the EF-14 trial [43]. Further treatment options are limited, and more randomized trials are needed to prove the role of a re-surgery or a second irradiation. Since recurrence rates remain high (~90%), lots of studies have been conducted to investigate the mechanisms of tumor chemoresistance and the strategies to overcome it. Among the ABC transporters, P-gp has been considered one of the main causes of tumor chemoresistance [44] since the upregulation of P-gp transcription can be induced by anti-cancer agents, the mutation of p53, activation of Raf and the presence of DNA damaging agents [45]. According to recent literature, the BBB ECs and their battery of efflux transporters, in particular the high expression of P-gp, are responsible for the low penetration of anticancer drugs and for the difficulties in drug development for GBM [46, 47].

#### **4.1. Glioblastoma vascularization**

GBM is a highly vascularized tumor characterized by an abundance of disorganized microvessels and an aggressive proliferation of endothelial cells and pericytes. The prognosis of patients may also be conditioned by these vascular structural anomalies sustained by the accompanying, constant expression of a number of angiogenic factors. Four types of microvascular formations, as described by Birner and Chen [48, 49], were identified in glioblastoma: (1) microvascular sprouting, defined as delicate capillary-like microvessels resembling those seen during classic angiogenesis, evenly distributed throughout the major parts of vital tumor tissue at the tumor border; (2) vascular cluster, defined as distinct focal aggregations of microvessels; (3) vascular garland, defined as clustered vessels arranged in a garland-like formation, with endothelial proliferation, frequently located around necrotic tissue; and (4) glomeruloid vascular proliferation, defined as clustered vessels ensheathed by hyperplastic endothelial cells and pericytes. However, despite their structural differences, a common histopathological feature in a large part of these tumor vessels is enhanced vascular permeability due to loss of the normal BBB, clinically highlighted by the tumor-associated brain edema and by contrast-enhancement on MRI scans.

## **4.2. Glioblastoma Blood-Brain Barrier/Neurovascular Unit**

The GBM microenvironment consists of highly specialized niches, such as the perivascular, the hypoxic-perinecrotic and the invasive niche at the tumor border, and each of them display different BBB properties. In the tumor center, excessive vascular growth, that is accompanied by severe hypoxia and necrosis due to elevated oxygen demands, leads to prominent BBB defects. At the tumor border, invasive glioma cells migrate along vessels that still have a mostly intact BBB. Neoplastic cells in early disease can survive within the perivascular space of the tumor NVU, and later remodel, destroy and produce new vasculature with aberrant BBB/NVU components. They function by providing traction support for migration utilizing proteins such as collagens, fibronectin and vitronectin, thereby promoting survival, proliferation, and maintaining multi-potency via the activation of pathways including TGF-beta, cytokine, Notch, Sonic Hedgehog and extracellular matrix (ECM) signaling, and facilitating radio- and chemotherapy resistance. At the same time, neoplastic cells produce and secrete additional ECM molecules, growth factor proteins, and matrix metalloproteinases (MMPs) to loosen the ECM allowing invasion [50]. This complex molecular crosstalk between tumor cells and the NVU components enhances tumorigenic processes and disrupts normal NVU functions. Displacement of normal astrocyte endfeet promotes vascular ‘leakiness’ through downregulation of tight junction (TJ) protein expression [51]. Beyond NVU disarray by individual or small clusters of neoplastic cells, larger tumors promote neovascularization to ensure adequate oxygen and metabolic support; these newly induced vessels have an abnormal NVU structure and function. GBM cells release proangiogenic growth factors such as vascular endothelial growth factor (VEGF), which activates VEGF receptors on endothelial cells, inducing them to sprout and extend into the tumor mass. Newly formed vessels can lack structural components of the NVU such as adequate pericyte coverage, normal EC walls and TJs, as well as normal basement membrane composition, and have variable permeability.

### **4.2.1. P-glycoprotein and tumor endothelial cells**

The study of P-gp expression at the tumor BBB ECs has been one of the focuses of neuro-oncological research in the last decades. In 1994 Tanaka and colleagues [52] reported that P-gp production was limited to endothelial cells from both human glioma and normal brain specimens, whereas no production of P-gp was noted in human glioma xenografts by IHC with anti-P-gp monoclonal antibodies. P-gp was preferentially expressed on the luminal side of the ECs in glioma by immunoelectron microscopy. In 1999 Sawada and colleagues [53] confirmed that all their 16 samples of GBM had a positive immunoreactivity for P-gp in endothelial cells and the signal was present mostly on the luminal membrane and was usually situated in the inner layers of glomeruloid proliferation. Similar data were also observed by Fattori in 2007

[54], who demonstrated that ECs of the newly formed capillaries in 16 of 39 glioblastomas (41%) expressed P-gp. However, in the literature there are many contradictory results on the expression of the MDR1–P-gp. In fact, it is not rare for controversial indications about the presence or the expression level of P-gp to emerge from different laboratories upon examination of identical tumor specimens as, for example, reported in the work of Nabors and colleagues [55]. Distinct aspects, including the use of weakly discriminating monoclonal antibodies and/or unsuitable techniques and procedures, contribute to generate such differences. After a first detection of P-gp expression in adult human cerebral cortex by immunofluorescence confocal microscopy using a monoclonal antibody to P-gp (JSB-1 clone) we tested, on HT 1080 cultured cells, colon carcinoma tissues, and human brain sections, four mouse monoclonal antibodies against P-gp, clones MRK16, C219, C494, and JSB-1 [15, 27]. Hence, in accordance with the results, we extensively used clone C494, whose effectiveness is very high on both cell cultures and tissue sections, throughout the study on human GBM (personal communications\*/\*\*). The applied IHC protocol, together with high resolution confocal microscopy, allowed the detection of a strong P-gp signal and an adequate subcellular detail in the analysis of glomeruloid microvascular formations in GBM (Figs. 4A-I; 5A-D). While on these sections a small number of glomeruloid vessels actually displayed a detectable P-gp on the endothelial luminal cell membrane (Fig. 4A-C), a higher number of vessels was observed to be characterized by a low or near absent EC P-gp-reactivity (Figs. 4D-I and 5A-D). These data were collected during previous studies on glomeruloid vessels in GBM [56, 57] and are in agreement with the detected expression profile of tight junction proteins and metabolic transporters at the endothelial lining [56]. In fact, in anaplastic astrocytoma and glioblastoma samples, the BBB-specific glucose transporter isoform 1 (Glut-1) consensually decreases where occludin, claudin-1, and claudin-5 detection appears significantly reduced [56]. There are two main hypotheses that could explain the discrepancy between the data on tumor EC P-gp: it was suggested that the presence of an intact BBB, associated with a high degree of cell-to-cell contact, could be essential for the endothelial expression of P-gp, thus where there is more breakdown of the BBB, as in the central part of GBM tissue, there may be less presence of P-gp, or else, as a second or complementary explanation, the human samples analyzed in the different studies may belong to patients already treated with chemo- or radiotherapy. It must be considered that temozolomide can itself induce the expression of MDR efflux transporters in GBM, as reported by Munoz et al. and Perazzoli et al. [58, 59]. Moreover, as suggested from Jodoin and colleagues, the interaction with caveolin-1 negatively regulates P-gp efflux activity, thereby promoting intracellular drug accumulation [29], while the expression of caveolin-1 is associated with sensitivity to temozolomide and this could be due in part to functional interactions between P-gp and caveolin-1 [60].

\* Virgintino D, Frei K, Girolamo F, Errede M, Roncali L. A confocal microscopy study of human glioblastoma microvessels by cell- and angiogenic-specific markers. Abstract volume of the 7<sup>th</sup> Cerebral Vascular Biology International Conference; 2007 June 24-28; Ottawa, Canada. \*\* Virgintino D. In-depth analysis of P-gp and NG2 in glioblastoma. 12<sup>th</sup> International Conference on Cerebral Vascular Biology; 2017 November 28 – December 1; Melbourne, Australia.

#### 4.2.2. P-glycoprotein and tumor astrocytes

Astrocytes, centrally positioned between neurons and ECs, extend their perivascular endfeet to the surface of CNS microvessels, becoming a fundamental cell component of the NVU and providing ~99% of the abluminal vessel coverage. Tumor astrocytes, associated with GBM microvessels, are involved in cancer cell growth, tumor invasion and infiltration of glioblastoma stem cells (GSCs), breakdown of normal BBB, and limited response to radiation or chemotherapy [61]. Studies on co-culture with astrocytes demonstrated that in these conditions GSCs appear less responsive to chemotherapy [62, 63]. On the other hand, GBM cells can actually activate the tumor astrocytes, to promote GBM invasion in the healthy tissue [61]. GBM-activated astrocytes induce in GBM cells a high level of expression for periostin and serglycin genes, increasing cell proliferation and glioblastoma cell growth in both cell lines and in a patient-derived cultures [64]. Accordingly, the interest in the role of tumor astrocytes in gliomas has increased and recent studies demonstrate that tumor-associated astrocytes can undergo different reactive transformations. Astrocytes, selectively purified from tumor specimens by advanced immunoprecipitation methods, reveal a reactive state marked by IL10 and IFN $\gamma$  response resulting in JAK/STAT pathway activation. This subtype of reactive astrocytes has been demonstrated to significantly contribute to the anti-inflammatory environment, resulting from mutual interactions between microglia and astrocytes that express CD274 (PDL1, Programmed Death ligand 1) as the target for the JAK/STAT pathway, which provides immune suppression through the PD1-PDL1 axis and promotes tumor growth and invasion [65, 66]. Reactive astrocytes can also promote *in vitro* a subtype-shift of glioblastoma towards the mesenchymal phenotype, driving mitogen-activated protein kinases (MAPK) signaling as well as increased proliferation and migration [67]. In addition, evidence suggests that astrocytes can be co-opted by neighboring tumor cells through the release of extracellular microvesicles (MVs), which communicate molecular and signaling changes and induce astrocytes to promote tumor growth [68] (see below). However, it has been suggested that also cultured astrocytes shed MVs, loaded with angiogenic growth factors [69]; if tumor-associated, activated astrocytes behave similarly they might intervene in tumor neo-angiogenesis and support tumor growth. This is supported by the observation of an enormous number of MVs released in GBM samples by both GSCs and tumor, perivascular astrocytes (Fig. 5A-D) (unpublished observations). Astrocytes associated to GBM vessels, as part of the deviant, tumor NVU, might also be implicated in GBM MDR. In the work by Guo and colleagues [70] on pediatric astrocytoma, P-gp has been found to co-localize with S-100 protein on the perivascular astrocyte endfeet. It is therefore worth noting that on our double immunostaining with GFAP and P-gp, tumor astrocytes, unlike their normal counterpart [15, 27], express high levels of P-gp (Figs. 4 and 5). The stronger P-gp immunosignal has been detected in the vicinity of the tumor vessel wall, where



P-gp and GFAP are seen to co-localize on bodies and processes of tumor astrocytes (Figs. 4C, F, I and 5A, C). At this site P-gp-reactive astrocytes can easily contribute to GBM MDR, supporting the ECs in their efflux activity, when endothelial P-gp is expressed (Fig. 4A-C), and/or taking the efflux activity of ECs, when these cells express low or no levels of P-gp (Fig. 4D-I).

#### 4.2.3. P-glycoprotein and glioma stem cells

While the multidrug resistance transporters have been extensively studied at BBB level and in tumor-associated astrocytes, less attention has been paid to the role of GSCs in the perivascular niche as a part of the P-gp ‘defense line’. In the last two decades, P-gp expression in glioblastoma cells has been extensively demonstrated in different cell lines as well as in rat models of GBM or in tumor xenografts [71-76] and some data confirmed that the percentage of MDR1-positive cells in surgical specimens increases with tumor malignancy from low-grade glioma to GBM [77, 78]. In the last years, many studies in the oncology research field have shed a light on the presence of a subpopulation of cancer cells possessing stem-cells properties. In glioblastoma, despite the fact that many of these cells may be derived from astrocytes post-epigenetic changes, rather than from a normal stem cell line, the term GSCs identifies a sub-population of tumor cells that exhibit proliferative and self-renewal properties, as well as multi-lineage differentiation. GSCs, with their high activity of DNA-repairing systems, the constitutive activation of pro-survival and anti-apoptotic pathways and the presence of hypoxic and growth factor-rich niches, appear to overexpress P-gp and many other transporters that drive the efflux of different chemotherapeutic agents (e.g. doxorubicin, etoposide, carmustine, vincristine, and temozolomide) [79], thus being mainly responsible for glioblastoma resistance to the available chemotherapies and to radiation [80-82]. In fact, in 2009, Nakai and colleagues [83] demonstrated, *in vitro*, a pool of MDR cells with both enhanced expression of both CD133 (a neural stem cell marker) and overexpression of P-gp in a surgical specimen of human glioblastoma xenografts. In our observation of *ex vivo* human GBM specimens, with high resolution confocal microscopy, we confirmed that the perivascular front of GFAP/P-gp-reactive tumor astrocytes is massively ‘infiltrated’ by P-gp-expressing glioma stem-like cells, closely packed around the vessel wall and adjacent to the vessel basement membrane edge (Fig. 4 and Supplementary Fig. 2). An *in vitro* study demonstrated that GSCs release MVs/exosomes, which may drive molecular changes and induce a tumor-promoting and/or tumorigenic phenotype in normal human astrocytes [68]. Actually, the observed P-gp-reactive perivascular GSCs seem to release massive amounts of P-gp-stained MVs/exosomes in the surrounding microenvironment and toward the vessel lumen (Fig. 5 and Supplementary Fig. 2). Further extensive studies on the shedding of GSC-derived P-gp-positive MVs are needed to provide data on the possible biomarker value of P-gp MVs

and on their possible activity as long distance conveyors able to deliver MDR transporters to drug-sensitive cells [84, 85].

## **5. P-GLYCOPROTEIN IMMUNOLocalIZATION ON RADIAL-GLIA-LIKE NEURAL PROGENITOR CELLS**

In the late 1990s, the role of radial glia cells (RGCs), classically described as guides for migrating neurons during neurogenesis, and then as astrocyte precursors during gliogenesis, was radically revised and these bipolar cells, characterized by a long basal process, a body localized in the ventricular zone (VZ), and an apical, short process abutting the ventricular surface, were recognized as neural progenitor cells (NPCs) [86, 87]. RG-like NPCs have been found in many regions of the developing brain, where they eventually give rise to neural stem cells (NSCs) in the adult subventricular zone (SVZ) [88, 89]. RG-like NPCs can be distinguished from their neuroepithelial progenitors (NEPs) by the expression of specific astroglia markers, including (in humans) the intermediate filament protein, gliofibrillary acidic protein (GFAP). Interestingly, although rodent radial glia do not express GFAP, the 2.2 kb element of the human promoter of the *GFAP* gene, termed *gfa2*, is transcriptionally active also in murine radial glia [90]. RG-like NPCs are self-renewal cells that comprise pluripotent progenitors as well as neuronal- and glial-restricted cell precursors [91]. It has been suggested, and genetically confirmed, that astrocyte-like NSCs, present in human adult SVZ, as a probable developmental reminiscence of RG-like NPCs, could be the cells of origin that enclose the driver mutations of human GSCs and may promote human GBM and GBM regeneration and recurrence [92]. Alternatively, astrocytes that derive from RGCs detached from the ventricle during neurohistogenesis, ‘outer RGCs’, may undergo de-differentiation to RG-like NPCs and initiate GBM [93, 94]. In fact, an important question in glioblastoma is whether differentiated brain cells, such as astrocytes, can de-differentiate and become tumor astrocytes and eventually glioblastoma stem cells. Glioma-like cells have been successfully generated from primary human astrocytes by combined transduction of defined genetic factors, thus confirming that mature human astrocyte can de-differentiate and transform into cancer cells and tumor-initiating cells [95]. Moreover, experiments carried out by stereotaxic injection of oncogenic lentivector demonstrate that mature astrocytes may de-differentiate, express progenitor markers and, in this progenitor state, initiate and maintain the tumor progression [96]. Results on P-gp cellular immunolocalization, carried out in human developing telencephalon, seem to confirm data on a possible astrocyte de-differentiation. In fact, the P-gp immunosignal was revealed on GFAP-reactive RG-like NPCs, located in the proliferative VZ of the future neocortex [97] (Fig. 6A-C) and was demonstrated to progressively decrease during cortical histogenesis (Fig. 6 D-I), in parallel with the last waves of neuroblast migrations, while the ‘outer’ RGCs and the derived astrocytes did not stain for the efflux transporter.

## 6. CONCLUSIONS

The attempts to inhibit P-gp efflux activity both by competing at the P-gp sites and by direct inhibitors, despite considerable *in vitro* successes, have failed on *in vivo* human glioblastoma as well as in experimental paradigms of the tumor, and have been associated to complex pharmacokinetic issues [98], thus also asking for the pursuit of better understanding of P-gp cell distribution in each potential site responsible for tumor MDR. Even if interest among the cancer research community is increasing, the role of activated astrocytes remains largely unknown, including the possible involvement of perivascular astrocytes in additional and/or auxiliary mechanisms of glioma chemoresistance. In human GBM, P-gp expressed by ECs may be a negligible component of the tumor MDR. Instead, tumoral perivascular astrocytes may de-differentiate and resume a progenitor-like P-gp activity becoming MDR cells and contributing, together with perivascular P-gp-expressing GSCs, to the MDR profile of the GBM NVU. Correlations between P-gp immunolocalization in normal developing and adult brain, highlighting the ‘progenitor’ status of perivascular tumor astrocytes, suggest the GBM NVU as the site for chemoresistance established by multiple cell lines.

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## **COMPETING INTERESTS**

The authors declare that they have no competing interests.

## **CONSENT FOR PUBLICATION**

All authors read and approved the final manuscript.

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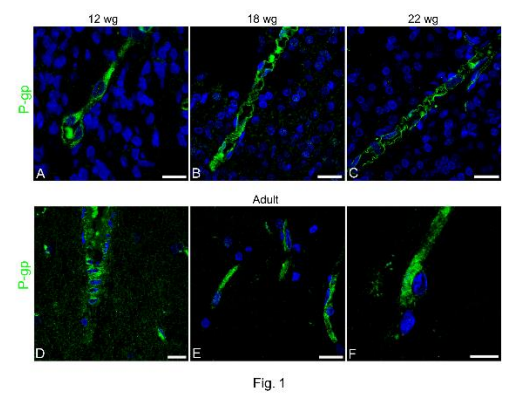


Fig. 1

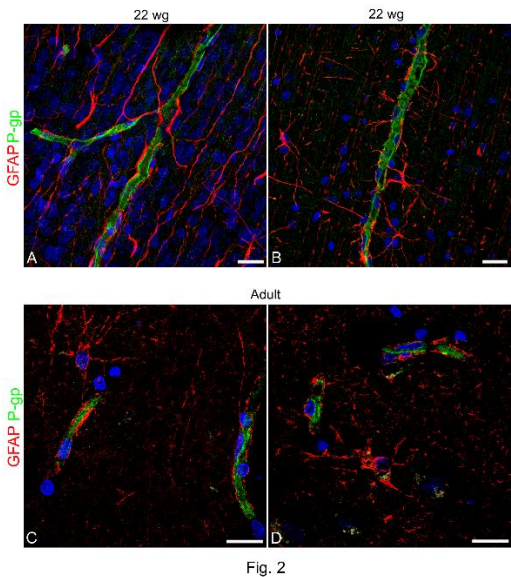


Fig. 2

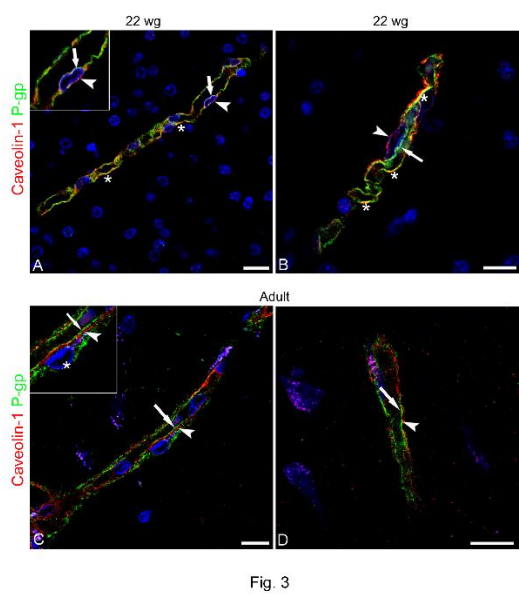


Fig. 3

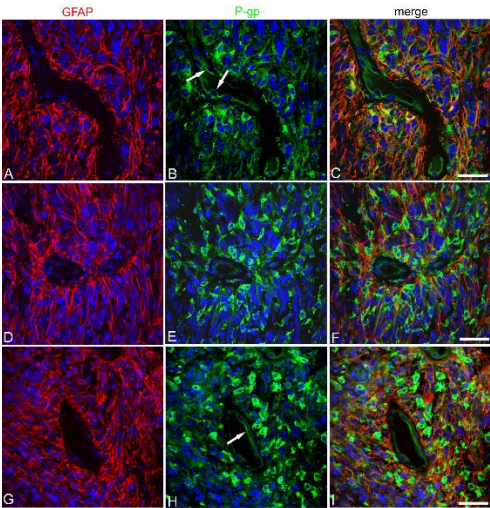


Fig. 4

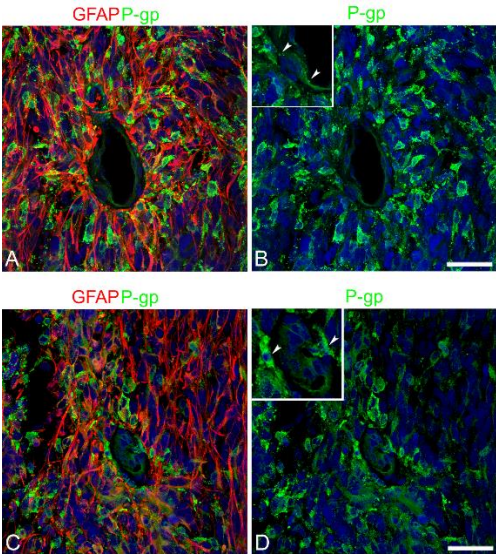


Fig. 5

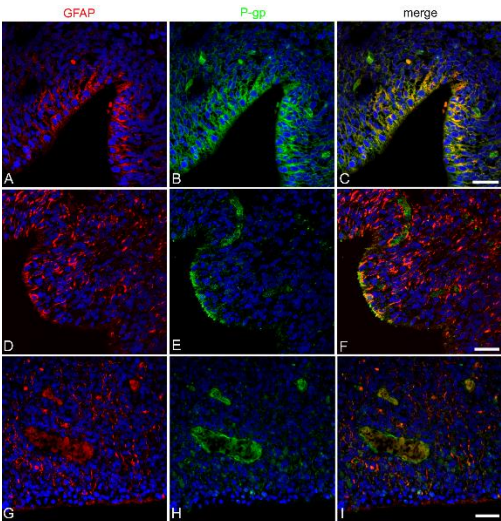
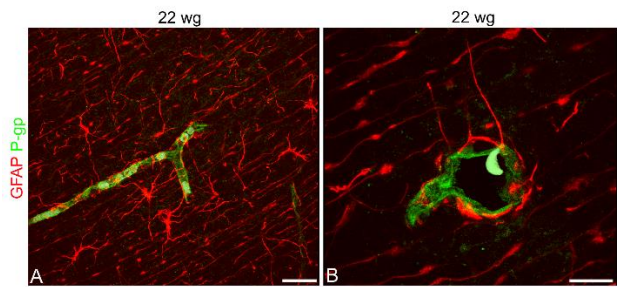
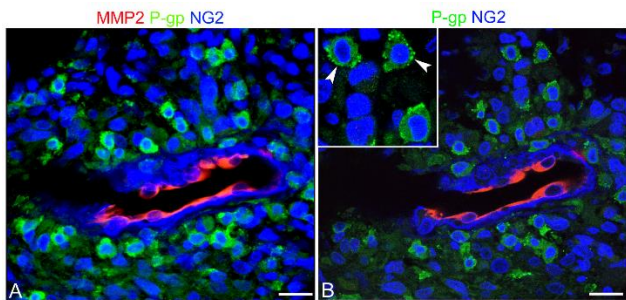


Fig. 6



Supplementary Fig. 1



Supplementary Fig. 2

## FIGURE LEGENDS

Fig. (1). P-gp immunolabeling of human fetal telencephalon at 12 (**A**), 18 (**B**), and 22 (**C**) weeks of gestation (wg), and of human adult brain **D-F**. **A**) P-gp staining appears uniformly distributed in the endothelial cytoplasm of the developing cerebral cortex microvessels at 12 wg, while it changes in a strong, linear P-gp pattern in the cortex of 18 and 22 week-old fetuses (**B**, **C**). **D**) P-gp staining of smooth muscle cells of a penetrating arteriole in the adult cerebral cortex. **E**, **F**) P-gp staining of endothelial cells cortex microvessels. Nuclear counterstaining TO-PRO-3. Scale bars, A-F 10  $\mu$ m.

Fig. (2). GFAP/P-gp double immunolabeling of human fetal telencephalon at 22 weeks of gestation (wg) (**A**, **B**) and of human adult brain (**C**, **D**). **A**, **B**) During development, P-gp is detected on the microvessel endothelial cells of both cortex (**A**) and subcortical (**B**) layers, whereas radial glia fibers and astrocytes and their perivascular endfeet appear stained by GFAP. **C**, **D**) P-gp-reactive microvessels of the adult cortex are surrounded by GFAP perivascular astrocytes. Nuclear counterstaining TO-PRO-3. Scale bars, A-D 10  $\mu$ m.

Fig. (3) Caveolin-1/P-gp double immunolabeling of human fetal telencephalon at 22 weeks of gestation (wg) (**A**, **B**) and of human adult brain (**C**, **D**). **A**, **B**) On endothelial cells, P-gp prevails on the luminal cell membrane (*arrow*), while caveolin-1 is detected on the abluminal one (*arrowhead*) (see also the *inset* in **A**); the two molecules are also seen to colocalize on some endothelial tracts (*asterisks*). **C**, **D**) The same distribution is observed on adult brain microvessels with caveolin-1 primarily localized on the abluminal endothelial front (*arrowhead*) and P-gp concentrated on the luminal one (*arrow*) (see also the *inset* in **C**); the *inset* in **C** shows a P-gp-reactive pericyte (*asterisk*). In *purple*, lipofuscin autofluorescence. Nuclear counterstaining TO-PRO-3. Scale bars, A-D 10  $\mu$ m.

Fig. (4). GFAP/P-gp double immunolabeling of human glioblastoma (**A** - **I**). **A**, **D**, **G**) GFAP immunostaining shows numerous tumoral astrocytes crowded in close vicinity of the tumoral vessel wall. **B**, **E**, **H**) In **B** an example of tumor endothelial cells stained by P-gp on their luminal front (*arrows*), whereas in **E** and **H**, P-gp is detected at very low levels on the endothelial cells, where it rarely marks the luminal plasma membrane (see *arrow* in **H**); numerous, strongly P-gp-stained glioma cells surround the tumor vessels. **C**, **F**, **I**) On the merged images, P-gp co-localizes with GFAP on perivascular tumoral astrocytes (*yellowish fluorescence*). Nuclear counterstaining TO-PRO-3. Scale bars, 15  $\mu$ m.

Fig. (5). GFAP/P-gp double immunolabeling of human glioblastoma (**A** - **D**). **A**, **C**) A continuous layer of GFAP/P-gp immunostained tumoral astrocytes (*yellowish fluorescence*) in close vicinity of tumoral vessels; all the perivascular area is infiltrated by P-gp-reactive cancer cells, which release a large amount of P-gp-stained microvesicles (MVs). **B**, **D**) P-gp-stained MVs are better seen on the single P-gp channel; note in **B** (*inset*) MVs very close to the endothelial lining and within the vessel lumen (*arrowheads*), while in **D** (*inset*) P-gp-positive glioma stem cells are close to the endothelial cells (*arrowheads*). Nuclear counterstaining TO-PRO-3. Scale bars, 15  $\mu$ m.

Fig. (6). GFAP/P-gp double immunostaining of human fetal telencephalon at 12 (**A-C**), 18 (**D-F**), and 22 (**G-I**) weeks of gestation (wg). **A-C**) In the ventricular zone (VZ), P-gp co-localizes with GFAP on the cell body of RG-like NPCs. **D-F**) P-gp/GFAP RG-like NPCs reduces at 18 wg. **G-H**) At 22 wg, the VZ does not stain for P-gp, except for periventricular microvessels. Nuclear counterstaining TO-PRO-3. Scale bars, 15  $\mu$ m.

#### SUPPLEMENTARY FIGURE LEGENDS

Supplementary Fig. (1). GFAP/P-gp double immunolabeling of human fetal telencephalon at 22 weeks of gestation (wg) **A**) A P-gp-positive microvessel of the subcortical plate surrounded by GFAP-positive/P-gp-negative perivascular and parenchymal astrocytes. **B**) P-gp-reactive endothelial cells and GFAP-positive perivascular astrocyte endfeet that do not stain for P-gp. Scale bars, A 25 $\mu$ m, B 10  $\mu$ m.

Supplementary Fig. (2). MMP2/P-gp/NG2 triple immunolabeling of human glioblastoma. (**A**) A stack of confocal planes and (**B**) a single confocal plane. **A**) Metalloproteases 2 (MMP2)-positive/P-gp-negative endothelial cells (*red*) of a tumor vessel, the proteoglycan NG2-positive vessel basal membrane (*blue*) [57], and numerous P-gp-reactive glioma stem cells (green). **B**) The single plane, from the same stack shown in **A**, reveals the subcellular detail of P-gp localization on the plasma membrane of cancer stem cells (*inset, arrowheads*). Nuclear counterstaining TO-PRO-3. Scale bars, A, B, 15  $\mu$ m.