Graphene-induced transdifferentiation of cancer stem cells as a therapeutic strategy against glioblastoma

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Review

Graphene-induced trans-differentiation of cancer stem cells as therapeutic strategy against glioblastoma

Costanza Martelli, Alice A. K. King, Thomas Simon, and Georgios Giamas

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30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52

Graphene

Graphene oxide

table of contents graphic

82x34mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Graphene-induced trans-differentiation of cancer stem cells as therapeutic strategy against glioblastoma

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Abstract

Glioblastoma (GBM) is an extremely malignant tumor of the central nervous system, characterized by low response to treatments and re-occurrence. Such therapeutic resistance is believed to arise mostly from the presence of a sub-population of tumorigenic stem cells, known as cancer stem cells (CSCs). In addition, the surrounding microenvironment is known to maintain CSCs, thus supporting tumor development and aggressiveness. This review introduces a novel therapeutic strategy involving the stem cell trans-differentiating ability of graphene and its derivatives. Graphene distinguishes itself from other carbon-based nanomaterials due to an array of properties that makes it suitable for many purposes, from bioengineering to biomedical applications. Studies have shown that graphene is able to promote and direct the differentiation of CSCs. The present review also provides a general outlook of the potential side effects (e.g. cell toxicity) that graphene could have. Additionally, potential usage of graphene in GBM treatment represents a challenge in respect to its administration method. Overall, this report will discuss certain potential graphene-based therapeutic strategies targeting CSCs for future effective GBM treatments.

Keywords

Cancer, differentiation therapies, graphene, central nervous system

1. Glioblastoma stem cells

1.1. Introduction to glioblastoma

Glioblastoma (GBM), further defined as a grade IV glioma, is one of the most prevalent primary brain tumors. It is characterized by a low survival rate and high malignancy, with patient life expectancy after diagnosis ranging between 15 and 19 months¹. Three main molecular subtypes of GBM have been identified, namely classical, proneural and mesenchymal, with the latest known as the most aggressive one. Classical GBM is mostly distinguished by EGF-R amplification together with the loss of chromosome 10 and amplification of chromosome 7². The proneural subtype is mainly characterized by overexpression of PDGF-RA and oligodendrocyte development genes such as OLIG-2 along with TP53 and IDH1 mutations. The mesenchymal subtype instead presents a greater astrocytic and mesenchymal marker expression, such as CD44 and MET, together with NF1, TP53 and PTEN mutations²⁻³.

GBM is characterized by high resistance to the classical therapeutic strategy associating surgery followed by concomitant chemotherapy and radiotherapy. Consequently, the tumor often recurs, in an even more aggressive fashion^{1-2, 4-6}.

1.2. Cancer stem cell characteristics and isolation

As in other types of solid cancer, GBM therapeutic resistance is thought to be due to the presence of cancer stem cells (CSCs) in the tumor bulk⁷. CSCs are a subpopulation of cells that present the characteristics of normal stem cells (SCs) within a specific intra-tumoral niche⁸⁻¹¹. Interestingly, such characteristics are driven by key transcriptional factors analogous to those of normal SCs, such as Bmi1, c-Myc, Krueppel-like factor 4 (KLF4), NANOG, octamer-binding transcription factor 4 (OCT4), spalt like transcription factor 4 (SALL4), SRY-related HMG-box 2 (SOX2) and signal transducer and activator of transcription 3 (STAT3)⁷. Consequently, developmental pathways such as the Notch, Sonic hedgehog (Shh) and Wnt ones, have been shown to be essential to GBM SC maintenance. Notch activation, potentially through the extracellular protein Tenascin-C, has been involved in supporting GBM SC stemness but also GBM tumors' invasiveness¹²⁻¹³. Therefore, Notch blockade combined with TMZ treatment and radiotherapy has been recently reported to affect the CSC population in GBM while improving survival *in vivo*. In addition, Yang *et al.* showed that inhibition of histone deacetylase 6 (HDAC6) leads to the inactivation of the Shh/Gli1 signaling pathway and consecutive GBM SC differentiation, while limiting tumor cell proliferation¹⁴. *Via* the activation of NANOG expression and concomitant down-regulation of the BMP-4 activity, the Shh/Gli pathway drives stemness maintenance, proliferation and therapeutic resistance in GBM SCs¹⁵⁻¹⁶. Similarly, as Wnt is a key player in normal development of the CNS, aberrant activation of the Wnt-affiliated pathways are directly associated to GBM

progression. The link between the Wnt pathway activation and GBM SC maintenance involves several different genetic and epigenetic mechanisms. Notably, both the canonical and non-canonical (β -catenin dependent and independent signaling, respectively) Wnt pathways are known to be directly linked to the EMT process in GBM SCs, underlying the cells self-renewal, migration and therapeutic resistance, via the activation of factors such as LEF1, Snail, Twist and ZEB1¹⁷. Moreover, Wnt3a can stabilize the epigenetic regulator KDM4C through the inhibition of the GSK3-dependent protein kinase R activity, hence avoiding the regulatory phosphorylation and consecutive ubiquitination. Such mechanisms then allows KDM4C to epigenetically activate the expression of Wnt target genes that are crucial for survival of GBM cells. Nevertheless, specific GBM SC sub-populations might individually depend on different pathways, as the Wnt/ β catenin signaling has been observed to be required for self-renewal maintenance in only a subset of CSCs¹⁸.

CSCs were originally isolated using cell surface markers such as CD133 and stage specific embryonic antigen-1 (SSEA1), normally used to purify normal neural SCs^{1,7}. Accordingly, CD133⁺ cells in GBM have been shown to be able to initiate new tumors *in vivo*¹⁹. Nevertheless, the reliability of CD133 as a marker for CSC isolation has now become object of debate. It has been demonstrated that CD133⁻ cell subpopulations display stem cell properties and the ability to initiate tumor development *in vivo*, as well as they can give rise to more aggressive tumors than CD133⁺²⁰⁻²¹. Therefore, there is a need for more specific CSCs markers that would allow both isolation and better therapeutic targeting^{4,22}. In this aim, the more specific aldehyde dehydrogenase 1 (ALDH1) marker can be used in combination with CD133 as it shows high levels of activity in precursor cells of GBM. Additional markers such as CD44 and ABC transporters are utilized as well^{8,23}.

1.3. Cancer stem cells in glioblastoma therapeutic resistance

Radiotherapy associated with the alkylating agent temozolomide (TMZ) is now the standard therapeutic strategy against newly diagnosed GBM. While DNA repair pathways have been widely described as being directly involved in GBM cell resistance to TMZ, additional resistance mechanisms seem specific to GBM SCs. Indeed, SCs resistance has been specifically related to drug efflux transporters, such as the ATP-binding cassette super-family G member 2 (ABCG2)²⁴. In addition, signaling directly involved in the maintenance of stemness in CSCs, including the c-Jun NH2-terminal kinases (JNK), SOX2 and Wnt/ β catenin pathways, also seems linked to chemoresistance mechanisms in GBM. Interestingly, the JNK and Wnt/ β catenin pathways have been reported to be directly linked to the expression of O-6-Methylguanine-DNA Methyltransferase (MGMT), a well-described TMZ resistance factor in GBM. Indeed, JNK inhibition has been shown to improve TMZ sensitivity in GBM²⁴. In the same way, researchers have observed that inhibiting the Wnt signaling could potentiate the GBM response to TMZ. Recent work also linked decreased

CD133, Nanog and SOX2 expression in GBM to reduced stemness and MGMT expression, consecutively leading to improved TMZ response ²⁵.

In addition, CSCs have also been reported to affect treatments specificity and consecutive efficacy through their potential capacity to generate high cellular heterogeneity in GBM tumors ^{3, 5-6}. Indeed, the asymmetrical division of CSCs that results into highly differentiated daughter cells allows diverse sub-populations of CSCs to co-exist in the same GBM region. Such differentiation is responsible for the tumor's heterogeneous phenotype, as shown in **Figure 1**, making specific cancer cell targeting very challenging ⁴.

Furthermore, it appears that GBM SCs do not only intrinsically resist to classic cancer treatments, but also reinforce their sub-population preservation in response to them. Indeed, DNA repair mechanisms, such as pathways involving key DNA repair kinases ataxia-telangiectasia mutated (ATM), Rad17, checkpoint kinase 1 (Chk1) and Chk2, have been reported as specifically activated in GBM SCs following radiotherapy ²⁶.

Moreover, close interactions with their direct microenvironment help sustain the GBM SCs' survival and renewing ²⁷. The GBM microenvironment is characterized by high, yet imperfect, vascularization, a hyaluronic acid (HA)-rich extracellular matrix (ECM) and presence of various cell types, namely macrophages, astrocytes, fibroblasts and endothelial cells, which, altogether, directly supports GBM growth ²⁸⁻³⁰. The highly vascularized niche at the core of GBM represents an extremely favorable environment for CSCs by promoting their survival, hence maintaining GBM heterogeneity and aggressiveness ³¹⁻³³. A precise tuning of the surrounding microenvironment thus appears critical to the GBM SCs phenotype ³⁴.

1.4. Differentiation therapy for glioblastoma treatment

Theoretically, being both the origins and key drivers of tumor progression, CSCs accordingly have many features in common with normal neural SCs ³⁵⁻³⁷. Hence, CSCs have been shown to be capable of forming neurospheres *in vitro* and differentiating into glial or neuronal cell lineages ³⁸. Recent publications have shown potential clinical benefits from triggering CSCs differentiation in GBM treatment. For example, dibutyryl cyclic AMP (dbcAMP) has been recently reported to trigger GBM cell differentiation into astrocytes through reversing the Warburg effect. As CSCs massively rely on anaerobic glycolysis, authors suggested that such an anti-Warburg effect strategy could induce CSC differentiation *in vivo*. As revealed by transcriptomic and proteomic data, Xiang *et al.* thus showed that the cAMP treatment induced an important mitochondrial biogenesis, *via* the cAMP response element-binding protein (CREB)-PPAR γ coactivator1 α (PGC1 α) signaling, leading to a metabolic reprogramming in favor of oxidative phosphorylation. Such shift eventually promotes astrocytic differentiation of GBM stem cells as evidenced by an increase of GFAP expression in

targeted cells, consequently inducing tumor growth inhibition in GBM xenografts models and patient-derived GBM samples ³⁹. In a different way, an all-trans retinoic acid therapeutic strategy, that has already shown efficacy in treating leukemia, could induce asymmetric division of glioma SCs, suggesting potential differentiation ⁴⁰. Furthermore, epigenetic modifier oligo-fucoidan (obtained from brown seaweed) has been reported to be able to suppress GBM cell proliferation while promoting cell cycle arrest at the G1 or S phase as well as inducing mRNA expression of differentiation markers, such as OLIG2 (oligodendrocyte), MAP2 (neuron) and GFAP (astrocyte). Authors described the differentiating activity of oligo-fucoidan to be linked to its repressive effect on DNA methyltransferases-1, -3A and 3B (DNMT1, -3A and -3B), thus causing the demethylation and consecutive expression of the p21 gene. As p21 is known to be a tumor suppressor and cyclin-dependent kinase (CDK) inhibitor, the differentiating effect of oligo-fucoidan appeared to be, at least partially, due to cell cycle arrest induction. Importantly, Authors also observed that normal astrocytes were not affected by such treatment, suggesting specificity to GBM cells ⁴¹.

As opposed to the use of such chemical compounds and inhibitors, biomaterials have been lately considered for fine tuning of the tumor microenvironment in the post-surgery cavity in order to trigger the differentiation of any remaining CSCs into non-tumorigenic/normal cells ⁹. Based on data from regenerative medicine, designing biomaterials could then be used to improve cancer treatments ⁴²⁻⁴³. Recent reports indeed detailed the differentiation of neural SCs into cells of glial lineages in materials such as HA hydrogels or poly-ethylene glycol matrices ⁴⁴⁻⁴⁵. In the same way, other devices such as 3D thermogels or poly (ϵ -caprolactone) microfibers, as they have been reported to have a pro-differentiation potential on normal neural stem cells, could also have anti-cancer applications in the near future ⁴⁶⁻⁴⁷. Therefore, through further addition of different conformations and gradients of compounds such as cytokines, growth factors or oxygen, researchers are confident they could regulate and control CSC differentiation and consecutive drug resistance ⁴³. Finally, use of biomaterials has also been studied for improving local delivery of specific drugs targeting CSCs. This way, Reguera-Nuñez *et al.* engineered an implantable poly(lactide-co-glycolide)-based microsphere encapsulating a heparin-BMP-7 nanocomplex to force GBM SCs differentiation into glial cells ⁴⁸.

In this context, evidence for the feasibility of the utilization of a biomaterial called graphene, a biocompatible scaffold component capable of influencing the growth, proliferation and differentiation of CSCs cultures *in vitro*, has been recently obtained ^{38, 49-50}.

2. Graphene

2.1. Graphene as a biocompatible scaffold

Graphene is a single sheet of graphite with a one-atom-thick hexagonal lattice, composed of a layer of carbon atoms, that makes it an extremely thin, yet strong, and a good conductive material⁵⁰. This nanomaterial presents a large surface area, high mobility of electrons at room temperature, good thermal conduction, elastic stiffness, as well as biocompatibility with several cell lines *in vitro*. Along with the ease of functionalization for specificity of interaction, all those parameters make graphene a remarkable material⁵¹⁻⁵². These physicochemical characteristics of graphene are currently extremely useful in clinical settings and research, as they are used for regenerative medicine and tissue engineering, including regeneration of neuronal networks in neurological diseases⁵³.

Given the strong dependence of CSCs' behaviors on their microenvironment, selecting the right biomaterial with appropriate physical and chemical properties to culture them *in vitro* is of fundamental importance⁵⁴⁻⁵⁵. Yet, graphene has been proven to be able to replicate the SCs' optimal physiological microenvironment, underlying its ability to help induce stem cell to grow and differentiate *in vitro*⁵⁶. Nevertheless, various physical forms and derivatives of graphene can differently affect stem cell actions⁵⁵.

Graphene is inherently a 2D material, even though it can be assembled into 3D structures. A common approach for tissue engineering application is to develop 3D foams⁵⁵. The main discernment that needs to be made between 2D and 3D models is that the former, although useful for traditional imaging methods, present a few challenges in mimicking the necessary structure, while the latter have been shown to be extremely well adapted for the *in vitro* culture of CSCs^{34, 57}.

Altogether, such artificial matrices thus need to be produced in respect to the brain ECM properties such as stiffness, degradability, and pore sizes³⁴. The ECM pore size is estimated to be 50nm, and the brain stiffness has been shown to be lower than the one observed in GBM⁵⁸⁻⁵⁹. In accordance with these data, stiff 3D graphene foams were reported as more efficient than soft ones at maintaining CSCs in an active state, increasing their proliferation and differentiation rate. Stiffness of the scaffold also affects CSCs morphology *via* alterations in their cytoskeleton⁵⁴⁻⁵⁵. In addition, 3D graphene foams also present an elevated porosity, specific surface area and a rough surface, displaying optimal characteristics to successfully imitate the brain ECM⁶⁰. Accordingly, Ma *et al.* recently showed that neural SCs grown on stiff graphene foams would adhere better and proliferate more than on soft foams while showing an increased astrocyte differentiation, as confirmed through the enhanced GFAP expression authors observed (**Fig. 2**). Authors also noticed that neuronal differentiation was reduced on stiff graphene foams as opposed to soft ones, thus underlying the importance of the material fine tuning in order to orientate cell differentiation⁵⁵.

2.2. Graphene's derivatives

In addition to different physical forms, graphene is also available as different chemical derivatives, namely graphene oxide (GO) and reduced graphene oxide (rGO) ⁶¹⁻⁶³. GO contains several functional oxygen-containing groups, such as carboxyls, hydroxyls and epoxides, which make it water-soluble and extremely beneficial for cell adhesion (**Fig. 3**) ⁶⁴. Furthermore, GO is flexible as well as being mechanically strong, combined with a nano-porous surface that promotes and supports the *in vitro* growth of CSCs ⁵⁶. GO is significantly cheaper and easier to fabricate in large quantities than pristine graphene, making it more attractive for broader applications. The differences between graphene and GO are summarized in **Figure 3**.

Graphene derivative rGO is also made of oxygen-containing functional groups together with the properties characteristic of GO, such as flexibility, mechanical strength and surface nano-porosity. However, due to reduction of oxygen functional groups, conductivity is improved while the surface of the rGO substrate is negatively charged, thus improving potential cell adhesion, as compared to 2D graphene film ^{61, 63}. The combination of these properties makes rGO substrates an extremely cytocompatible scaffold, which encourages CSC proliferation and differentiation *in vitro* to an even higher level than GO. Accordingly, Guo *et al.* reported that neural SCs grow around rGO fibers where they establish a structural network and expand rapidly. In addition, through Tuj1 and GFAP labelling *in vitro*, authors showed a stronger neuronal differentiation over glial differentiation in rGO structures, which is critical for neuronal regeneration applications ⁵⁴. Moreover, rGO has been shown to increase the expression of neuronal differentiation markers in neuroblastoma cells through mechano-transduction pathways ⁶⁵.

In a similar way, Chen *et al.* (2012) compared the effects of culturing induced pluripotent SCs (iPSCs) on a glass surface with culture on a graphene substrate or on a GO substrate. Data showed that iPSC proliferation and adhesion to graphene substrates was not different to culture on glass ⁶⁶. On the other hand, cells grown on GO instead showed improved adhesion and proliferation, which might be due to the presence of oxygen-containing functional groups making GO more hydrophilic. In addition, while the graphene substrate kept the cells in an undifferentiated state, GO promoted the differentiation of the SCs ^{9, 66}.

Nevertheless, it is thought that there could be negative outcomes resulting from the interaction between graphene and SCs, such as oxidative stress, as a result of reactive oxygen species (ROS) production ⁶⁷. Other potential effects of cytotoxicity of graphene substrates are damage to the cell membrane, fragmentation of DNA, apoptosis and chromosomal aberrations ⁶⁸. A study by Ren *et al.* (2016) thus showed toxicity in the CNS of zebrafish provoked by GO that had been dispersed in water. Authors report that such toxicity manifested itself through appearance of Parkinson's disease-like symptoms ⁶⁹. Still, the levels of induced toxicity and damage seem to be correlated to the method

of graphene administration chosen, in addition to the level of functionalization of the substrate, as well as its size and dose. Indeed, toxicity seems to arise only following a chronic exposure to graphene and its derivatives ⁷⁰. Moreover, studies showed that oral administration of rGO nano-sheets caused a decrease in activity of locomotion, while no neurotoxicity appeared in rats injected intravenously with rGO flakes ^{67, 71}.

3. Use of graphene for GBM differentiation therapy

3.1. Impact of graphene on molecular pathways specific to CSC maintenance

Pluripotency genes such as SOX2, OCT4 and NANOG are known to be responsible for the stem cell self-renewal properties ⁷¹. SOX2, for instance, is directly involved in preventing differentiation of neural SCs into neuronal lineages ⁴. Accordingly, graphene has been reported as affecting such key pathways, supporting its potential as a novel differentiation therapeutic ⁷²⁻⁷³.

In this aim, Gurunathan and Kim (2017) combined GO with silver nanoparticles (GO-AgNPs), to treat SH-SY5Y neuroblastoma cancer SCs. Through monitoring neurite outgrowth, the authors noticed that the GO-AgNPs treatment could induce branching of long neurites as well as network formation. They also reported that GO inhibited CSCs pluripotency by down-regulating the cells' self-renewal capacity, reporting an increase in neuronal differentiation rate and a decrease in the expression of stem cell markers such as OCT3, SOX2, NANOG and KLF4. In parallel, authors observed an increase of the expression of key neuronal markers such as β III-tubulin or fox-1 homolog 3, in the SH-SY5Y cells following treatment with GO-AgNPs. Interestingly, as GO-AgNPs significantly increased ROS levels in the neuroblastoma cells, authors suggested that oxidative stress could drive the observed neuronal differentiation, as similarly described in other studies ⁷⁴. They also demonstrated an increase of the expression of key signaling pathways, such as Akt, ERK1/2, P53 or P21 signaling, known as being implicated in cell cycle control and cell differentiation ⁴⁹.

Graphene and its derivatives, through inhibition of the CSCs pluripotency and self-renewal ability, would therefore lead to the forced differentiation of the CSC population, hence preventing tumor's treatment resistance and relapse ⁷¹.

Furthermore, CSCs have been shown to maintain their self-renewal and differentiation properties using the support of vascular endothelial cells within the vascular niches of GBM ¹⁰. Such stemness maintenance is partly due to the release of nitric oxide by endothelial cells, thus activating the Notch pathway in CSCs ^{69, 75}. This pathway has been described as preventing the neuronal differentiation of early progenitor SCs in certain brain regions but also hypothesized to direct the stem cell fate towards different cellular lineages in the mature brain as, for instance, its activity is inhibited to stimulate neuronal differentiation ^{22, 76}. In addition, recent studies interestingly observed that the Notch pathway is also directly involved in the stem cell interactions with the direct micro-

environment, for instance through regulating the SCs niche's size. The Notch pathway thus appears central in the fate of neural SCs and CSCs.

Accordingly, a study by Fiorillo *et al.* thus reported that GO managed to provoke the differentiation of CSCs, therefore reducing their tumor-sphere formation capabilities. Authors suggested such effect could take place through inhibition of the Wnt, Notch and STAT3 signaling pathways, as well as a reduced nuclear factor erythroid 2-related factor 2 (NRF2)-dependent anti-oxidant response⁹. As the authors observed that the GO flakes used were larger (5 to 20 μ M) than the CSCs, it was suggested that the flakes couldn't be internalized with the cells. Consequently, authors concluded that the GO flakes had to interfere with the signaling pathways directly on the cellular surface, which is where signaling pathways involved in maintaining stemness, such as the Notch and STAT3 pathways, are also initiated. The STAT3 pathway is indeed involved in the growth, division and apoptosis of cells and it was observed that by genetically knocking it down *in vivo*, the tumorigenicity would diminish. However, unlike Notch, interference with the STAT3 pathway is not specific to CSCs, which could lead to damage to normal SCs as well⁴. Further studies on the effect of graphene and its derivatives on the different processes linked to CSCs specific signaling pathways are thus fundamental. They could indeed be of extreme importance in the development of a treatment aiming at controlling the proliferation of CSCs in tumors such as GBM^{9, 22}.

3.2. Specificity of graphene towards CSCs

The concept of developing targeted treatments that could make GBM less aggressive and prevent its reoccurrence is extremely promising, but there needs to be the certainty that normal brain cells and neural SCs are not affected in the process.

Furthermore, recent reports have documented high cytotoxic, anti-proliferative and anti-migration effects of graphene on GBM cells⁷⁷⁻⁷⁸. Wierzbicki *et al.* indeed showed that graphene oxide nanoplatelets (nGO) could reduce the U87 and U118 GBM cell lines migration and invasion *in vitro*, showing only low cytotoxicity. Such effects were described by the authors to be due to alterations in the adhesion-dependent EGF-R/Akt/mTOR and β catenin signaling pathways. They suggested that the impact of graphene on the cell surface morphology might have led to the decrease in EGF-R phosphorylation they observed. In the same way, the cell ability to adhere to the ECM was disrupted following treatment with nGO, altogether linking graphene interactions with the cell surface to direct anti-tumor effects⁷⁷. In a more extensive way, Szczepaniak *et al.* reported that graphene and numerous of its derivatives could show anti-tumor abilities on GBM cells *in vitro*. Their highly valuable data described the effects of the GN/ExF, rGO/Term, rGO/ATS and rGO/TUD graphene derivatives, each carrying different oxygen content, on GBM cell viability, metabolic activity, apoptosis and cell cycle dynamics. Even though every compounds were able to decrease cell

metabolic activity and cell viability consecutively, the highest effect was seen following treatment with rGO/TUD, while cell apoptosis was affected the most by GN/ExF, most likely through disturbance to the mitochondrial membrane potential. According to the authors, the observed effects of high concentrations of graphene derivatives on GBM cell viability can also derived from physical and biological damage done to the cell membrane ⁷⁹. Furthermore, as pure graphene (GN/ExF) showed some of the highest cytotoxic effects, as compared to oxygen-containing derivatives, authors stressed that even a minimal oxygen content can decrease the pro-apoptotic capabilities of graphene and its derivatives. However, oxygen-containing derivatives appeared to have a better affinity for GBM cells than pure graphene, supporting a better targeted effect. Overall, authors thus concluded that the surface and function of graphene are essential parameters directly impacting its physico-chemical properties and biocompatibility. In addition, they added that functionalizing rGO with amino acids, such as arginine or proline, can reduce the cytotoxic effects as well as improving anti-tumor capabilities of graphene derivatives by avoiding agglomeration ⁸⁰. Altogether, these reports suggest that fine tuning of the graphene properties when used to treat GBM tumors could allow targeting specific cell populations, including CSCs, while avoiding disturbing normal/stromal cells.

Accordingly, Verre *et al.* (2018) observed that graphene and its derivatives had little toxicity on glial cells while enhancing the rate of stem cell differentiation ⁸¹. Furthermore, a study by Gurunathan and Kim (2017) showed that a GO substrate can lead to oxidative stress in basal epithelial A549 cells only at high concentrations and leaving the cell membrane intact ⁴⁹. Finally, Fiorillo *et al.* (2015) also confirmed the GO substrate's specificity for only targeting CSCs in a study considering GBM together with breast, prostate, ovarian, lung and pancreatic cancer. Indeed, authors observed that GO could inhibit tumor-sphere formation *in vitro* while leaving non-stem cancer cells and normal fibroblasts unharmed ⁹. Authors there also observed that the different normal cells in the body were not affected or only relatively affected by big flakes (5-20 μ m) of GO substrate. In addition, it appeared that normal SCs were instead only affected by graphene substrates in regards to their differentiation rate, which was promoted towards different cellular lineages, but were not subject to any toxicity ^{9, 49}. Similarly, Szczepaniak *et al.* recently reported that the cytotoxic effects of graphene flakes they could observe on U87 GBM cells were less harmful to stromal Hs-5 cells ^{78, 82}. Yet, in follow-up studies, the authors reported a toxic effect of pristine graphene on both GBM and stromal cells, thus pointing out the importance of choosing the right material modification for specifically targeting CSCs ⁸³. Furthermore, in a parallel study, the same authors observed that GO could down-regulate genes of the mitochondrial oxidative phosphorylation (OXPHOS) complexes in GBM, thus limiting cancer cell invasion capabilities. As CSC metabolism and survival is highly dependent on OXPHOS, as compare to normal cells or proliferating tumor cells, authors suggest that the reported effects on cancer expansion might be due to a disruption of the CSC functioning ⁸².

3.3. How is graphene ‘delivered’ to GBM?

For all the aforementioned reasons, it has been suggested that GO flakes could be implanted to the tumor site following the initial surgery to cleanse the tumor site from the CSCs that might have survived the GBM mass excision⁹. According to the authors, such strategy would theoretically prevent the re-occurrence of GBM by directly targeting the CSCs subpopulation responsible for the tumor onset⁹.

In the same way, graphene could also be administered intravenously or orally^{9,67}. Yet, in this case, potential side effects and graphene interaction with the BBB need to be investigated. Graphene could indeed provoke inflammation due to its interaction with lipids and proteins⁶⁴. It has been reported that large graphene flakes were able to trigger a greater inflammatory response as compared to small flakes⁸⁴⁻⁸⁵. It thus appears that graphene preparation and modifications have a strong influence on the effect of this biomaterial on immune response⁶⁴. Nevertheless, graphene can also cause haemolysis as a result of engaging with components of the blood stream and aggregation⁸⁶. In addition, thrombotic response has also been reported in reaction to blood administration of graphene. Further functionalization of graphene could potentially help avoid such negative side effect⁸⁷. Bioaccumulation of graphene is another limiting factor for proper graphene delivery to the desired target. Again, proper functionalization of graphene might help go around the issues⁸⁸. For all these reasons, GO substrates seem to be preferred to pristine G for biomedical/clinical applications⁶⁷.

Different approaches, such as ultrasounds application and photo-thermal treatment, are therefore being considered for improving delivery. Furthermore, linkage of graphene substrates to ligands recognized by specific receptors has been proposed as a potential strategy for precise cell targeting. For instance, in order to target breast cancer cells Zhang et al. used graphene conjugated to folate, which is known to be overexpressed at the surface of some cancer cells^{64, 89}. For similar reasons, the use of graphene as a drug nano-carrier to directly target GBM's CSCs had recently been examined. Again, the presence of the BBB nevertheless challenges the realization of such strategy^{50, 90}.

Yet, Yang *et al.* (2014) investigated a technique in which the tight junctions of the BBB are reversibly opened through ultrasounds application. This way, nano-sheets of GO successfully reached the brain after they had been loaded with miRNA let-7, a tumor suppressor, and injected into mice's tail⁹¹. Moreover, recent studies assessed the efficacy of graphene functionalization with molecules specifically interacting with constituents of the BBB, such as the Tat protein or dextran, thus allowing crossing to the SNC⁹²⁻⁹³.

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4. Final remarks

Treatment resistance and tumor re-occurrence are conferred to GBM mainly by the presence of the highly resistant subpopulation of CSCs found in the inner chore of the tumor mass and their direct interactions with the brain microenvironment. Targeting genes and signaling pathways underlying the CSC maintenance could thus be effective for the therapeutic treatment of GBM. The nanomaterial graphene and especially its derivatives, namely GO and rGO, are a promising tool to achieve this goal. Indeed, using them as *in vitro* scaffolds for CSCs from GBM showed extremely interesting results. For instance, rGO substrates not only increase both proliferation and differentiation rate, but also ‘re-direct’ the differentiation of the CSCs towards neuronal lineages rather than glial ones. In theory, such graphene-triggered differentiation of GBM CSCs would then prevent the tumor from re-growing after the mass is surgically removed. It is therefore of extreme importance to carry out further studies to confirm this hypothesis and, consequently, develop a technique to safely deliver the graphene substrate to the GBM site in the brain (**Table 1**).

Furthermore, it should be mentioned that another application for graphene substrates that seems to be valid for the treatment of cancer is the use of graphene nanoparticles for phototherapy⁹⁴. In that case, the graphene nanoparticles are utilized to generate accumulation of enough heat to be able to affect the tumorigenic cells they are bound to. In this method, near-infrared (NIR) radiations are used to excite graphene nanoparticles, hence converting the radiations into vibrational energy, resulting in cancer cell necrotic death or apoptosis. Tis way Markovic *et al.* (2011) managed to limit U251 glioma cells survival *in vitro*^{67, 94}.

Altogether, it appears that the use of graphene substrates, through their multiple hypothetical applications, could indeed provide with highly valuable therapeutic options for the treatment of GBM, with consecutive positive effects on patients’ quality of life.

Authorship

AK, TS and GG conceived the idea. CM, AK and TS did the literature research. CM, AK, TS and GG wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Table 1

Reference	Cell type	Graphene material	Set up	Observed effects	Pathways & markers
Wierzbicki <i>et al.</i> , 2017 [77]	GBM cell lines	GO nanoplatelets	<i>In vitro</i>	GO nanoplatelets reduced GBM cell line ECM adhesion, migration and invasion with low cytotoxicity	Alterations to EGF-R/Akt/mTOR and β catenin signaling pathways
Szczepaniak <i>et al.</i> , 2018 [79]	GBM cell line	Graphene and rGO derivatives (rGO/Term, rGO/ATS, rGO/TUD)	<i>In vitro</i>	Every compounds decreased cell metabolic activity and viability; best effect on cell metabolic activity was observed with rGO/TUD; data suggested best targeted effect against GBM cells with oxygen-containing derivatives.	Alterations of the cell membrane and mitochondrial activity
Szmidt <i>et al.</i> , 2019 [82]	GBM cell line	Graphene, GO & rGO	<i>In vitro</i>	Anti-proliferative and anti-migratory effects of GO on GBM cells	Down-regulation of the mRNA expression of mitochondrial OXPHOS genes
Jaworski <i>et al.</i> , 2019 [83]	GBM cell line	Graphene platelets	<i>In vitro</i> & <i>In vivo</i>	Dose-dependent cytotoxicity of graphene on GBM cells in vitro; Mass and volume reduction of GBM tumors in vivo following exposure to graphene platelets	Graphene exposure decreased mitochondrial functions leading to ROS formation, thus altering cell viability
Ma <i>et al.</i> , 2016 [55]	Neural SCs	Graphene foams	<i>In vitro</i>	Better adhesion, higher proliferation and astrocyte differentiation of neural stem cells on stiff graphene foams as compared to soft foams	Enhanced GFAP and GAP-43 expression, decreased Tuj1 expression in neural stem cells grown on stiff graphene foams as compared to soft foams
Guo <i>et al.</i> , 2017 [54]	Neural SCs	rGO microfibers	<i>In vitro</i>	Better adhesion, higher proliferation and neuronal differentiation of neural stem cells on rGO microfibers as compared to 2D graphene film and tissue culture plates	Enhanced Tuj1 expression in neural stem cells grown on rGO microfibers as compared to 2D graphene film and culture plates
Fiorillo <i>et al.</i> , 2015 [9]	Several CSCs including GBM SCs	GO flakes	<i>In vitro</i>	GO flakes inhibited tumor-sphere formation in CSC cultures while being armless to proliferating cancer cells and fibroblasts	Reduced Wnt, STAT3, Notch, NRF2, INF γ -STAT1, SMAD-TGF β signaling pathways

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Figure legends

Table 1: Evidence for application of graphene substrates towards glioblastoma treatment. Most relevant publications providing evidence for use of graphene substrates and derivatives in therapeutic strategies against GBM, including differentiation therapy. GBM: glioblastoma; GO: graphene oxide; rGO: reduced graphene oxide; ECM: extracellular matrix; EGF-R: epidermal growth factor-receptor; mTOR: mammalian target or rapamycin; OXPHOS: oxidative phosphorylation; GFAP: glial fibrillary acidic protein; GAP-43: growth associated protein-43; Tuj1: neuron specific class III beta-tubulin; SC: stem cell; CSC: cancer stem cell.

Figure 1: Hypothetic cancer stem cell origins in glioblastoma. GBM tumors are very heterogeneous at the cellular and molecular levels, with 3 different main subtypes, which are usually all present in the same tumor bulk. Such high heterogeneity suggests the existence of multiple potential CSC origins. As one of the most studied is the possible occurrence of cancer transformation in normal neural stem cells, GBM CSCs could also arise from more differentiated cells such as neural progenitors or astrocytes and oligodendrocytes. In addition, as the tumor expands and more genetic mutations accumulate, further potential CSCs can arise from differentiated cancer cells, from which new cancer cells of altered molecular background can arise, thus contributing to the tumor high heterogeneity (see Neftel *et al*, 2019). GBM: glioblastoma; CSC: cancer stem cell. *Servier Medical Art*.

Figure 2: Influence of graphene scaffold stiffness on neural stem cell behavior (based on Ma *et al.*, 2016). Fine tuning of graphene scaffolds can specifically orientate neural stem cell differentiation. Recent reports, including one by Ma *et al.* (2016), showed that neural stem cells show a better adhesion and proliferation on stiff graphene scaffold than on soft ones. In addition, authors observed that astrocyte differentiation increases with the scaffold stiffness while neuronal differentiation reduces. Accordingly, neural stem cells tend to rather differentiate into neurons on soft graphene scaffolds, as opposed to stiff ones. *Servier Medical Art*.

Figure 3: Graphene and Graphene oxide basic structures. GO contains several functional oxygen-containing groups, such as carboxyls, hydroxyls and epoxides, which make it water-soluble and extremely beneficial for cell adhesion. GO: graphene oxide.

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Graphene-induced trans-differentiation of cancer stem cells as therapeutic strategy against glioblastoma

Costanza Martelli, Alice King, Thomas Simon and Georgios Giamas