

Chapter 2

Connexins: Bridging the Gap Between Cancer Cell Communication in Glioblastoma

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Abstract Despite concerted clinical and research efforts, glioblastoma (GBM), the most prevalent primary malignant brain tumor, remains uniformly lethal. Like other advanced cancers, GBM is characterized by extensive cellular heterogeneity and is organized in a hierarchy with self-renewing, therapeutically resistant cancer stem cells (CSCs) at the apex. While communication between GBM tumor cells and their surrounding stroma supports tumor survival and expansion, the mechanisms behind direct cell-cell communication and its contribution to tumor growth have yet to be fully elucidated. In particular, the biological importance of intercellular communication between GBM tumor cells, including CSCs and non-stem tumor cells (NSTCs) has yet to be determined. Gap junctions (GJs) are specialized structures, composed of connexin proteins, allowing for the diffusion of small molecules and ions directly between the cytoplasm of adjacent cells, enabling them to respond to each other and external stimuli rapidly and coordinately. Connexins have been found to help promote tumor cell growth, invasiveness, and tumorigenicity, making them attractive anti-tumor targets. However a complete understanding of the function of connexins and GJs in GBM remains an area of active investigation. Here we discuss recent advances in connexin function as they relate to our understanding of cellular communication and malignancy in GBM.

Keywords Glioblastoma • Gap junctions • Connexins • Cancer stem cells

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Over the past century, major medical innovations have improved the standard of living for much of the developed world. Likewise, the average life expectancy has increased in many countries as a direct result of our continued efforts to understand the biology of disease and apply this knowledge toward the development of more efficacious therapeutic interventions. However, while mortality rates have decreased for many illnesses such as heart and cerebrovascular disease, little progress has been made in the fight against cancer, as death rates due to malignant neoplasms have remained steady over the past 50 years [1]. Primary central nervous system (CNS) tumors, in particular, are among the most dangerous malignancies, comprising only 2 % of all cancer diagnoses but accounting for a disproportionate rate of morbidity and mortality.

The most common aggressive of all primary CNS tumors is glioblastoma (GBM), accounting for 20 % of all intracranial tumors in the United States [2]. GBM is classified by the World Health Organization (WHO) as a grade IV glioma and is thought to arise from glial cells: non-neuronal cells responsible for maintaining homeostasis, forming myelin, and providing protection for neurons in the brain and peripheral nervous system [3]. Most GBM tumors (~90 %) appear to rapidly expand *de novo* mainly in elderly patients, without evidence of a precursor lesion, and are classified as primary GBM. However, a smaller fraction (~10 %) of secondary GBM tumors are thought to progress from low-grade astrocytomas, mainly in younger patients. Primary and secondary GBM tumors are largely indistinguishable histologically but differ in their genetic and epigenetic profiles. Additionally, secondary GBMs carry a vastly favorable prognosis compared to primary GBM diagnoses [4]. The histological criteria for GBM diagnosis include cellular pleomorphism, nuclear atypia, vascular thrombosis, microvascular proliferation, and necrosis with lesions displaying both intra- and inter-tumoral heterogeneity [4]. Currently, GBM is uniformly fatal, and treatment is only palliative, consisting mainly of maximal safe surgical resection, chemotherapy, and radiation. Independent prognostic factors include patient age, performance status, number of lesions, and extent of resection [5]. Despite aggressive therapy, median survival time after diagnosis is 12–18 months, while the 5-year survival rate remains at 5 % [6].

The high degree of invasiveness characterizing GBM is a major impediment for treatment, as surgical resection is often unable to remove the entirety of the tumor, leaving behind a population of infiltrating cancerous cells that egress away from the primary site [7]. Further surgical intervention is made difficult or impossible as the remaining tumor cells continue to migrate along myelinated axons, vascular basement membranes, or the subependyma to infiltrate anatomically critical structures in the brain and escape the reach of current therapeutics [8]. Likewise, due to the heterogeneous nature of GBM, the administration of chemotherapy and radiation may have deleterious consequences for patient survival. According to the stochastic model of tumor formation, all tumor cells are thought to possess the ability to propagate a tumor, with genetic cues dictating which cells drive tumor progression. Accordingly, a small number of tumor cells, through clonal selection, are thought to be capable of randomly developing resistance to current therapeutics. As such, treatment may inadvertently select for more aggressive tumor clones, helping

explain why recurrence is almost always inevitable following initial tumor debulking [9]. Recent clinical trials interrogating the efficacy of several antiangiogenic agents in conjunction with radiation and chemotherapy appear to reinforce the concept of clonal selection in GBM. Antiangiogenic therapy resulted in short-term tumor burden control and improved progression-free survival. However, overall survival was not impacted [10, 11], indicating the rise of a more aggressive, recurrent population of tumor cells following initial therapy.

The stochastic model is an attractive intellectual concept regarding the formation and maintenance of GBM. It proposes that tumors arise from a single clone, allowing for the sequential selection of progressively more malignant cancer cells. As tumor cells acquire additional mutations, some variants are destroyed while others are imparted with growth advantages, permitting clonal expansion. These clones become the predominant subpopulation until a more favored variant appears. Eventually, the acquired genetic instability and associated selection process results in a heterogeneous population of cancer cells making up a tumor [12]. However, this model does not take into account environmental factors and epigenetic variables that influence cell behavior independently of genomic control. As such, it is an incomplete and simplistic view of tumor biology, necessitating additional models to better represent the complex nature of GBM.

A surrogate hypothesis is the hierarchical model of cancer, which posits the existence of a cellular hierarchy within a tumor. A small sub-population of cells, referred to as cancer stem cells (CSCs), exist at the top of the hierarchy and are capable of propagating tumor heterogeneity. The hierarchical model accommodates the possibility that CSCs are capable of retaining responsiveness to external environmental cues, eliciting their genomically determined potential for self-renewal and recapitulation of the cellular diversity composing the bulk of the tumor [13]. However, the two models should not be considered to be mutually exclusive. The CSC hypothesis does not exclude stochastic selection or the acquisition of resistance by tumor cells, and instead, both models should be viewed complementarily to address the complexity of tumorigenesis [14].

Indeed, recent evidence has demonstrated the existence of CSCs crucial for GBM initiation and maintenance [15]. Unlike their rapidly proliferative non-stem tumor cell (NSTC) counterparts, CSCs are resistant to chemotherapy and radiation as a result of an increased DNA repair capacity [16, 17]. The exclusive ability of CSCs to self-renew and differentiate into multiple lineages is a major factor in GBM tumorigenesis and recurrence [14]. In light of their unique phenotype, CSCs have since been recognized as attractive targets for the development of novel, combinatorial GBM therapeutics aimed at eradicating both the bulk of the tumor as well as the resistant CSC population. However, prior to successful clinical translation, several challenges remain in the integration of CSC-specific interventions alongside current standard-of-care modalities. Among them are complications in the categorization of cellular differentiation states in GBM lesions, given that stemness is a dynamic property within a tumor. As such, CSCs have the capacity to differentiate into NSTCs while still retaining the ability to revert back to a CSC state in response to microenvironmental cues such as hypoxia [18], pH [19], and metabolism [20].

Additionally, CSCs share common gene-expression signatures as well as cell signaling pathways with neural progenitor cells (NPCs) [21], hampering the development of agents capable of destroying the former while preserving the latter.

While multiple cell types likely contribute to GBM growth, cancer cells display a remarkable ability to tailor and influence their microenvironment for the promotion of tumor growth, maintenance, and migration [22]. Physically, tumor cells are capable of remodeling their surrounding extracellular matrix (ECM), both through the production of matrix metalloproteinases (MMPs) and the degradation or synthesis of collagen ligands to facilitate invasion into the brain parenchyma. In addition, glioma cells apply physical stresses on the surrounding collagen matrix, as a result of traction forces exerted by individual cells and compressive forces generated by the expansion of the tumor bulk, to influence tumor cell proliferation and malignant outcome [23]. In addition, it is becoming more appreciated that GBM is not simply composed of small numbers of CSCs and their NSTC progeny but rather contains a proportion of host cells and tissue. The capability of normal tissue to directly influence tumor biology and vice versa should not be taken lightly as mutual interactions between neoplastic and non-neoplastic cells produces a local milieu, favoring tumor cell growth and immune escape. Tumor-associated cells in the GBM microenvironment, such as microglia, vascular cells, peripheral immune cells, and NPCs, also play important roles in the pathology of GBM, often exerting pro-tumorigenic effects [22].

Likewise, the host immune system is capable of interacting with GBM tumor cells. GBM CSCs are capable of driving tumor growth by actively attenuating immunosurveillance through the secretion or expression of immunosuppressive factors or by the recruitment of accessory cells, which locally suppress the immune response until tumors reach a size at which they surpass immune pressure, resulting in progression and malignancy. In addition, GBM CSCs are capable of recruiting multiple cell types with tumor-supportive phenotypes. In vitro, CSC-conditioned medium was found to increase monocyte migration compared to cell suspensions generated from GBM NSTCs [24]. Likewise, CSCs have been shown to secrete soluble colony stimulating factor-1 (sCSF-1), C-C motif ligand 2 (CCL2), and macrophage inhibitory cytokine 1 (MIC-1) to enhance monocyte infiltration into the tumor [24]. However, upon recruitment of peripheral monocytes into the tumor, the secreted sCSF-1 and CCL2 polarize them toward the immunosuppressive M2 macrophage phenotype, while MIC-1 simultaneously inhibits their phagocytic ability [24]. Moreover, recent work has revealed that CSCs are similarly capable of recruiting tumor-associated macrophages (TAMs) into the tumor microenvironment by secreting periostin, a protein normally thought to support the adhesion and migration of epithelial cells, through its receptor, integrin $\alpha_v\beta_3$ [25].

Based on these observations, GBM should not be thought of as one distinct entity residing in normal brain tissue but rather as an aberrant organ. Like normal organs, GBM tumors are composed of multiple cellular and stromal aspects working in concert for proper function, under physiological conditions, or malignancy, under neoplastic conditions. Additionally, both normal and cancerous cells must be able to interact and communicate with various, surrounding cell types to execute

biological functions at the tissue level that could not otherwise be accomplished [26]. The information exchanged between cells may involve direct cell-to-cell contact or the release of soluble mediators capable of acting in an autocrine or paracrine manner, depending on the nature of the signaling pathway. However GBM tumors do not frequently metastasize to other organs of the body and remain confined to the brain parenchyma, which is itself enclosed by the blood-brain-barrier, limiting cellular cross-talk across peripheral circulation. As a result, GBM tumors often histologically manifest as dense hypercellular masses with little room between individual cells. The close confines of the GBM microenvironment necessitates rapid tumor cell communication both between other tumor cells as well as with the surrounding stroma in order to coordinately respond to chemical and physical stimuli. Both the spatial limitations of the brain and the temporal need to quickly adapt to an ever-changing environmental milieu make it likely that GBM tumor cells rely on autocrine and paracrine signaling pathways through direct cell-cell contact as a means of communication. Gap junctions (GJs) represent a well-documented means of intercellular communication in various tissues. GJ-mediated communication has been demonstrated to be essential in normal embryonic development [27], electric coupling in cardiac muscle [28] and neurons [29], as well as in normal hematopoiesis [30]. Additionally, connexin expression in non-excitabile tissues has key roles in organ development [31], skeletal development [32], and growth control [33].

GJs are aggregates of intercellular channels composed of a family of 24 proteins, termed connexins, that allow the direct transport of cytoplasmic contents from cell to cell. Six co-oligomerized connexin subunits form a connexon, also known as a hemichannel. The connexin subunits making up a connexon can either be identical (homomeric) or dissimilar (heteromeric), although not all connexin subunits are capable of forming a functional hemichannel [34]. Two hemichannels on different cells are then able to dock and form a homotypic or heterotypic GJ channel, depending on the connexin isotype. A functional GJ channel allows for the diffusion of small molecules up to 1 kDa in size between the cytoplasm of adjoining cells. GJs favor the intercellular exchange of metabolites such as adenosine diphosphate (ADP), glucose, glutamate and glutathione [35], as well as second messengers such as calcium ions (Ca^{2+}), cyclic adenosine monophosphate (cAMP), and inositol triphosphate (IP_3) [36].

The expression of connexin proteins is both tissue specific and developmentally regulated, making the number of combinations of possible intercellular channels broad. The variability of connexin signaling also plays an important role in the physiological properties of the various gap junction hemichannels, including conductance and permeability [37]. Traditionally, connexin function has been linked to the formation of gap junction channels although it is becoming more appreciated that connexin hemichannels are capable of serving as aqueous pores permeable to ions and small molecules [38, 39], that link the intra- and extracellular compartments. In addition, dysregulated connexin expression has also been linked to at least ten distinct diseases, such as X-linked Charcot-Marie-Tooth disease [40], keratitis-ichthyosis-deafness syndrome [41], and oculodentodigital dysplasia [42]. The

importance of regulating connexin function in normal development may also underscore the role that aberrant expression plays in tumor formation and growth.

Historically, connexins have been thought to function as tumor suppressors in several animal models of cancer, including hepatoma [43] and thyroid tumors [44], as well as human carcinoma of the stomach [45], which was evidenced by a lack of electrical coupling between tumor cells. However, recent evidence has suggested that connexins may also promote tumorigenesis. Forcing connexin expression in both non-metastatic and metastatic tumor cells with no prior functional connexin activity was shown to decrease proliferation and cell migration [46], and promote a mesenchymal to epithelial transition [47]. Likewise, in several advanced cancers, GJ function was associated with invasion [48], intravasation [49], extravasation [50], and metastasis of tumor cells [51], facilitating late-stage disease progression. Gap junctions also participate in the “bystander effect” following radiation therapy in which cells that are not directly exposed to radiation but are in the vicinity likewise respond to the exposure and display significant levels of genetic change and lethality.

In the CNS, abundant connexin expression has been demonstrated in multiple cell lineages, including neurons, astrocytes, and microglia. Under physiological conditions, connexins are thought to be important in normal neurogenesis as well as neuronal electrical signaling [35]. Connexin subunit expression was detected at the very early stages of neural development, with connexin 43 (Cx43) and connexin 45 (Cx45) robustly expressed and essential for rat NPC proliferation and survival [52]. Follow-up studies demonstrated that embryonic NPCs possessed active gap junctions as, confirmed by dye-coupling studies, and that in the absence of essential growth factors, Cx43 overexpression was sufficient to preserve NPC self-renewal, which was otherwise compromised in differentiation-inducing conditions [53]. Along with preserving self-renewal, connexins also impact lineage commitment, with connexin 36 (Cx36) being important in the modulation of NPC differentiation into neurons and glia [54]. Reduced Cx36 expression decreased neuronal commitment, and overexpression restored neuronal differentiation along with oligodendrocyte commitment [54]. It has recently been appreciated that connexins may also function to impact invasion as well as cellular signaling programs through interaction with scaffolding proteins via their cytoplasmic tails [55]. There is evidence for each of these aforementioned functions in the developing brain. NPCs utilize Cx43 and connexin 26 (Cx26) for tangential migration of newly-born neurons [56, 57]. While Cx43 reduction has a profound impact on rat and mouse NPCs, it was dispensable for human NPC function, and Wnt/ β -catenin signaling was activated in Cx43-reduced conditions [58], suggesting that the Cx43 may serve to suppress Wnt/ β -catenin signaling. These results also demonstrate the species difference that may exist for connexins and highlight the need for additional studies in multiple systems. The existence of connexin signaling in NPC maintenance is also important in the adult mouse brain, with connexin 30 (Cx30) and Cx43 found to mediate intercellular coupling between radial glial cells in the dentate gyrus. It was found that mice lacking Cx43 and Cx30 in radial glial cells showed complete inhibition of cell proliferation in the subgranular zone of the adult dentate gyrus [59]. In addition,

GJ-mediated communication was found to be crucial for several brain processes, including neuronal energy supply, electrical and chemical synapses, calcium waves, spatial buffering of K^+ and glutamate, maintenance of myelin and blood–brain barrier integrity [35].

While there is an established literature for the function of connexins during neural development, the role that connexins play in GBM and CSCs is only beginning to be investigated. Some studies have found that Cx43 is decreased in high-grade brain tumors [60], while others demonstrate that Cx43 is capable of conferring chemotherapeutic resistance to human glioma cells [61] through the upregulation of key pathways including the epidermal growth factor receptor [62]. Overall, this is reflected in the lack of a consensus for the pro- or anti-tumorigenic role for connexins in GBM, which has thus far mainly focused on data surrounding Cx43 [63]. Efforts have begun to interrogate the expression and function of connexins in CSCs. Similar to the early work in GBM, pro- and anti-tumorigenic roles have emerged and these may be model- and connexin subunit-specific. Overexpression of Cx43 was found to inhibit CSC self-renewal, invasiveness, and tumorigenicity via E-cadherin, which in turn influenced Wnt/ β -catenin signaling, increasing the latency of GBM tumors [64]. However, others have demonstrated that CSCs predominantly express Cx46, while their NSTCs express Cx43. As CSCs were differentiated, Cx46 was reduced while Cx43 increased, and targeting Cx46 rather than Cx43 was found to compromise CSC maintenance [65]. Along with serving as a functional regulator of CSC maintenance and possible driver of tumor progression, modulating connexins may be an adjuvant therapeutic approach. The rationale for this is the “bystander effect,” whereby damage generated from irradiating one cell may be passed to another via gap junctions. Recent work in a mouse medulloblastoma model used genetic approaches to downregulate Cx43 and demonstrated that gap junction-mediated communication is crucial for the transmission of radiation. Upregulation of Cx43 was found to cause tumor regression in the distal CNS, the area not exposed to direct radiation therapy, further supporting its anti-tumor role. Surprisingly, Cx43 was also found to be upregulated in non-targeted tissue following irradiation, which may allow for the transduction of potentially oncogenic signals to remote tissue through this “bystander effect” [66]. As evidenced, additional work is necessary to completely unravel the function of connexins in GBM, especially in the context of the “bystander effect” and therapeutic resistance. However, evidence strongly suggests that connexins are key regulators of GBM phenotypes and are emerging as attractive targets for potential therapeutic modalities aimed at reducing GBM invasiveness, proliferation, and lethality.

To better understand connexin biology and its role in disease, two non-specific pan-GJ inhibitors are currently being investigated in pre-clinical trials, Carbenoxolone (CBX) and 1-Octanol. CBX is currently approved in the clinical treatment of esophageal and mouth ulcers in the United Kingdom [67], while 1-Octanol is currently being interrogated for the treatment of essential tremors [68]. CBX, in particular, has been investigated in several advanced cancers, including thyroid [69], leukemia [70], and GBM [65] due to its minimal cytotoxic nature. Combinatorial treatment of primary human glioma isolates with CBX and

mesenchymal stem cells (MSCs) engineered to express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to enhance glioma cell death through the upregulation of death receptor 5. Importantly, dual therapy utilizing TRAIL and CBX prolonged mouse survival by ~27 % compared with control animals, suggesting a favorable clinical translation [71]. Likewise, both 1-Octanol and CBX were shown to prolong animal survival in intracranial and subcutaneous xenograft models of GBM. Interestingly, when GJ inhibition strategies were combined with temozolomide (TMZ), an additive effect on survival was seen, suggesting that the use of GJ inhibition strategies alongside current therapeutic modalities could positively impact patient outcome [65].

Targeting GJ-mediated communication in GBM and other cancers is emerging as an exciting prospective strategy with potentially translatable results. Specifically, the additive survival advantage that GJ inhibition, alongside chemotherapy, confers in animal models of GBM is particularly promising. However, several caveats remain to be addressed regarding both CBX and 1-Octanol before clinical trials are implemented. Both agents demonstrate remarkable efficacy for inhibiting GJs and tumor cell growth in vitro and in vivo. However, their mechanism of action is poorly understood. In particular it should be noted that they do not specifically block individual connexin subunits or GJs. Rather, they are pan-inhibitors, ostensibly blocking all connexin function and making it difficult to study which particular connexin subunits are involved in tumor biology. It is also important to note that blocking all GJs may have unintended off-target effects that need to be addressed before considering clinical trials. Additionally, the exact methods by which the agents inhibit connexin function is an ongoing area of investigation. It has been hypothesized that both CBX and 1-Octanol act on cell membranes to alter fluidity and disrupt the transmembrane domains of connexin proteins, rendering them inert. However, this explanation has yet to be fully investigated and remains speculative. The last, and possibly most important, point to consider regarding GJ inhibition is the exact mechanism behind tumor cell death after treatment with CBX or 1-Octanol. Several likely explanations for this phenomenon have therefore been proposed. As previously mentioned, GBM tumor cells exist in a closely packed microenvironment and communicate predominantly through cell-cell contact mediated by GJs. As such, tumor cells are better able to respond to external stimuli and escape damage from sources such as chemotherapeutics and radiation by exchanging information and rendering themselves less susceptible to perturbation. In addition, GJs may allow for the release of potentially lethal intercellular components, such as reactive oxygen species (ROS), generated in response to cell damage. Conversely, GJ hemichannels may also facilitate the uptake of molecules necessary to protect tumor cells from ROS-induced DNA damage. Recent work in normal hematopoietic stem cells (HSCs) has supported this concept, as Cx43 deficient HSCs displayed decreased survival and increased senescence as a direct result of their inability to transfer ROS to the hematopoietic microenvironment following myeloablation, demonstrating that Cx43 is able to play a protective role during stressful conditions such as hematopoietic recovery [72].

Even though the exact molecular mechanisms behind connexin signaling are only now beginning to be elucidated, the potential to disrupt GJs, and consequently tumor cells, by pharmacologically targeting connexins remains an attractive strategy in a field that has had limited clinical success over the past decades. However it is prudent to consider that connexins may have additional functions which have yet to be fully described. To this end, cytoplasmic partners have been thought to be capable of interacting with the intracellular domains of connexin proteins, providing a potential means of specifically targeting individual subunits. The ablation of one universal connexin may have unintended secondary effects or no effects at all, as compensatory mechanisms likely exist among various connexin proteins. Rather, GJ inhibition strategies should be contextualized in light of the overall tumor or, even more effectively, in light of the cell-of-origin of the tumor to target the root of the malignancy rather than the branches. Of paramount importance is the development of novel mimetic peptides or agents capable of disrupting individual connexin subunits to minimize the harm done to normal tissue in the course of treatment. Cancer therapy as a whole is moving away from a “one-size-fits-all” paradigm and towards a more individualized model. Targeting specific connexin subunits, depending on tumor subtype, is therefore complementary to the emerging trends regarding cancer care and should be considered for further attention. Additional work is also necessary to tease out the direct molecular mechanisms responsible for connexin signaling, but efforts are slowly beginning to concentrate on this line of inquiry. With careful methodology and proper animal models, elucidating connexin signaling has the potential to make a transformative impact for the development of therapies capable of improving the outcome of patients diagnosed with not only GBM but also other neoplasias for which little hope currently exists.

Conflict of Interest The authors wish to disclose that they do not have any relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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