

The role of ion channels in the hypoxia-induced aggressiveness of glioblastoma

Luigi Sforna¹, Marta Cenciarini¹, Silvia Belia¹, Maria Cristina D'Adamo², Mauro Pessia², Fabio Franciolini^{1*} and Luigi Catacuzzeno^{1*}

¹ Department of Chemistry, Biology and Biotechnology, University of Perugia, Perugia, Italy

² Faculty of Medicine, Section of Physiology and Biochemistry, Department of Experimental Medicine, University of Perugia, Perugia, Italy

Edited by:

Francesco Moccia, University of Pavia, Italy

Reviewed by:

Mohamed Trebak, State University of New York, USA

Alessandra Fiori Pla, University of Torino, Italy

*Correspondence:

Fabio Franciolini and Luigi Catacuzzeno, Department of Chemistry, Biology and Biotechnology, University of Perugia, Via Pascoli 1, I-06123 Perugia, Italy
e-mail: fabio.franciolini@unipg.it; luigi.catacuzzeno@unipg.it

The malignancy of glioblastoma multiforme (GBM), the most common and aggressive form of human brain tumors, strongly correlates with the presence of hypoxic areas, but the mechanisms controlling the hypoxia-induced aggressiveness are still unclear. GBM cells express a number of ion channels whose activity supports cell volume changes and increases in the cytosolic Ca²⁺ concentration, ultimately leading to cell proliferation, migration or death. In several cell types it has previously been shown that low oxygen levels regulate the expression and activity of these channels, and more recent data indicate that this also occurs in GBM cells. Based on these findings, it may be hypothesized that the modulation of ion channel activity or expression by the hypoxic environment may participate in the acquisition of the aggressive phenotype observed in GBM cells residing in a hypoxic environment. If this hypothesis will be confirmed, the use of available ion channels modulators may be considered for implementing novel therapeutic strategies against these tumors.

Keywords: hypoxia, glioblastoma multiforme (GBM), ion channels, aggressiveness, cancer

INTRODUCTION

Glioma is a major tumor involving glial cells in the central nervous system, and accounting for 35–50% of intracranial tumors in adults. Among them grade IV glioblastoma multiforme (GBM) has the highest incidence and shortest patient survival, with approximately three cases per 100,000 person-years and median survival of only approximately 10 months. The longest survival is achieved in patients who undergo resection followed by radiotherapy and chemotherapy, yet the median survival with this combined treatment is still only 20 months (Holland, 2001). Increasing malignancy of gliomas strongly correlates with insufficient blood supply, hypoxic areas and necrotic formations. Many hypoxic regions are found in GBM, including a central large necrotic core and multiple thrombotic foci surrounded by pseudopalisading cells migrating away towards more oxygenated areas (Rong et al., 2006; Amberger-Murphy, 2009). Hypoxia represents a major driving force for the development of tumor aggressiveness through increased invasion, resistance to apoptosis, chemo and radioresistance, and tumorigenic cancer stem cells development (Yang et al., 2012). While few of these effects are mediated acutely and “directly” by the hypoxic condition, in most cases hypoxia acts by gene expression changes promoted by hypoxia-induced factors (HIFs; Yang et al., 2012).

GBM cells express a number of ion channels, membrane proteins that allow the selective and controlled passage of ions along their electrochemical gradient. Most of them have a deregulated expression in GBM as compared to normal glial cells,

and the resulting alteration in membrane ion flux has important consequences in the acquisition of the features typical of the transformed cell. For example, the coordinated activity of the upregulated K⁺ and Cl⁻ channels generates transmembrane fluxes of the respective ions supporting membrane potential changes as well as cell volume changes promoted by the osmotically driven water flux. Volume changes are in turn strongly required for a number of basic cell functions, such as cell division and growth, migration and death (Akita and Okada, 2014). Likewise, Ca²⁺-selective ion channels, important modulators of the Ca²⁺-dependent enzymes, are also often upregulated in GBM, where they promote a number of important cell functions (Santoni et al., 2012). Thus, ion channels may control most of the cell functions also regulated by a hypoxic environment and may play important roles in the mechanisms leading to hypoxia-modulated GBM aggressiveness.

In several tissues, low oxygen levels regulate the expression or activity of many ion channels, including those controlling the aggressiveness of GBM (Shimoda and Polak, 2011). Since the initial description of a hypoxia-regulated ion channel (López-Barneo et al., 1988), numerous studies have been carried out indicating that a variety of ion channels are sensitive to oxygen, exhibiting changes in channel activity with acute hypoxia and alterations in channel expression with prolonged hypoxic challenge (chronic hypoxia).

Based on these observations we carried out a literature survey aimed at addressing the following two questions: (i) Are there GBM ion channels involved in the control of certain aspects of

cell aggressiveness that are also regulated by hypoxia? (ii) For these specific channels, do experiments performed in GBM or in other preparations have highlighted a hypoxic-mediated modulation of their expression or activity? This undertaking has been made bearing in mind the rationale that ion channels would be involved in the hypoxia-induced aggressiveness of GBM.

CHEMORESISTANCE

Among the chemotherapeutics most used in the treatment of GBM there are temozolomide (TMZ), carmustine (bis-chloroethylnitrosourea; BCNU) and doxorubicin (DOXO), all DNA alkylating agents causing DNA damage and cell death. Although the use of chemotherapeutics plays an important role in the combined treatment of GBM, it remains a challenge because of tumor chemoresistance (Haar et al., 2012). Among the many factors contributing to GBM chemoresistance is hypoxia, both with direct and indirect mechanisms. An obvious reason for this resistance is that hypoxic areas are often relatively far from blood vessels, preventing the chemotherapeutic agent from reaching its target (Vaupel et al., 2001). Another protective factor is the non proliferative nature of hypoxic cells, that makes them intrinsically resistant to the anticancer agents acting preferentially on cycling cells (Oliver et al., 2009). More detailed molecular research also suggests that among the targets of HIF-1 is MDRI, the gene encoding for P-glycoprotein, the drug transporter that strongly contributes to chemoresistance by a drug efflux activity (Comerford et al., 2002).

GAP JUNCTIONS

Several studies show an involvement in GBM chemoresistance for gap junctions, intercellular ion channels that allow the passage of small ions and molecules between neighboring cells (Simon and Goodenough, 1998; Goldberg et al., 1999; Fry et al., 2001). In astrocytes and astrocytoma cells gap junctions are formed by the connexin 43 (Cx43) subunits. It has been recently shown that increasing the level of Cx43 in human LN18 and LN229 glioma cells enhances resistance to TMZ, while knockdown of Cx43 sensitizes them to TMZ, demonstrating a fundamental role of gap junctions in the responsiveness to chemotherapeutics (Gielen et al., 2013). In these cells Cx43 alters mitochondrial apoptotic pathways by regulating the level of Bax2 and Bcl-2, as well as Cyt C release from the mitochondria following TMZ treatment (Gielen et al., 2013). In addition to its recognized role as channel protein, Cx43 has a “non-channel” mechanism of action involved in intercellular signaling (Goodenough and Paul, 2003; Naus and Laird, 2010), and this mode of action is thought to reverse the oncogenicity of human GBM cells (Huang et al., 1998). Interestingly, the Cx43-dependent TMZ resistance was found to depend on both its channel-dependent and -independent mechanisms, as demonstrated by using Cx43 channel-defective mutants (Gielen et al., 2013). Although the effects of hypoxia on GBM gap junctions have not been investigated, several studies in other preparations point to a possible modulatory role. In cardiac tissue and MDCK epithelial cells expressing Cx43, hypoxia rapidly activates Akt, which leads to Cx43 phosphorylation and to larger gap junctions (Dunn and

Lampe, 2014). Also in human mesenchymal stem cells Cx43 expression is increased by hypoxia (Grayson et al., 2007). Conversely in rat H9C2 and HL-1 cardiomyocytes hypoxia promotes Cx43 degradation (Severino et al., 2012; Wu et al., 2013). In cultured astrocytes Cx43 underwent dephosphorylation 30 min after hypoxia, and this was preceded by a strong reduction in gap junctional intercellular communication (Li and Nagy, 2000). Based on this evidence, it may be hypothesized that Cx43 participate in the hypoxia-induced chemoresistance observed in GBM.

Cl⁻ CHANNELS

A number of different Cl⁻ channels have been reported to be expressed in GBM cells and to be implicated in several cell functions, including chemoresistance (Sontheimer, 2004; Catacuzzeno et al., 2011, 2014; Cuddapah and Sontheimer, 2011). It was found that a BCNU-resistant subpopulation of GBM cells upregulated the ClC-1 intracellular Cl⁻ channel, whereas the unspecific Cl⁻ channel inhibitor DIDS synergistically augmented the apoptotic efficacy of BCNU (Kang and Kang, 2008). In addition, specific inhibition of ClC-3 expression by siRNA-mediated knockdown sensitized U251 cells to cisplatin-mediated cell death, through the downregulation of phosphorylated Akt (Su et al., 2013). Again, although no evidence is present in GBM cells for a hypoxia-induced modulation of Cl⁻ channels, in other preparations this modulation was found. For example, chronic hypoxia upregulates Ca²⁺-activated Cl⁻ channels in vascular smooth muscle cells (Sun et al., 2012), while acute hypoxia inhibited the swelling-activated Cl⁻ current (I_{Cl,Swell}) in cerebellar granule neurons (Patel et al., 1998). These data suggest a possible hypoxia-mediated modulation of Cl⁻ channels also in GBM, that can have a role in the hypoxia-induced chemoresistance.

MIGRATION/INVASION

GBM cells often invade adjacent normal brain tissue, and this infiltrative behavior makes complete surgical resection impossible, and tumor recurrences most frequent (Claes et al., 2007; Lefranc et al., 2009). Several studies have shown that hypoxia is a major promoter of GBM invasion. Under hypoxic conditions GBM cells increase both their bi-dimensional migratory ability, as tested by wound-healing assay, and their invasiveness on matrigel-coated transwell (Shen et al., 2013; Zhang et al., 2013). Both these types of hypoxia-induced migratory abilities were mediated by HIF-1, as demonstrated through knock-down experiments (Méndez et al., 2010; Esencay et al., 2013; Fujimura et al., 2013).

Cl⁻ CHANNELS

Several studies show that GBM cells over-express the Cl⁻ channels ClC-3 and the Ca²⁺-activated K⁺ channels KCa1.1 and KCa3.1, and their coordinated activity, promoting K⁺ and Cl⁻ transmembrane fluxes, supports cell volume and membrane potential changes necessary for GBM cell migration and invasion (Catacuzzeno et al., 2012; Turner and Sontheimer, 2014). ClC-3 channels have been shown to play an important role in cell migration and invasion (Olsen et al., 2003; Ernest et al.,

2005; McFerrin and Sontheimer, 2006), and found to be localized in invadopodia where they form protein complexes with other important modulators of cell invasion, such as matrix metalloprotease-2 and aquaporin-4 (Deshane et al., 2003). Given the previously mentioned evidence for a hypoxia-induced modulation of Cl^- channels, it may be hypothesized that a change in Cl^- channel expression or activity may have a role in the hypoxia-induced GBM invasiveness.

Ca^{2+} -ACTIVATED K^+ CHANNELS

KCa1.1 and KCa3.1 channels have also been shown to be required by several pro-migratory signals (Sontheimer, 2008; Sciacaluga et al., 2010; Catacuzzeno et al., 2011). KCa1.1 channels are expressed as a particular splicing isoform in GBM cells (the glioma BK, gBK; Liu et al., 2002), and are implicated in GBM invasion (Bordey et al., 2000; Wondergem and Bartley, 2009; Cuddapah and Sontheimer, 2011; Steinle et al., 2011). KCa3.1 channels are most important in GBM as they are virtually not expressed in normal, differentiated glial cells. Their presence could thus be exploited as an important diagnostic tool, and their pharmacological targeting could well represent a potential therapeutic strategy for this tumor (Catacuzzeno et al., 2012). It has been found that several recognized pro-migratory signals, such as fetal calf serum and SDF-1, activate KCa3.1 channels, leading to GBM cell migration (Sciacaluga et al., 2010; Catacuzzeno et al., 2011).

Several studies show that KCa1.1 and KCa3.1 channels can be modulated by chronic hypoxia. In human pulmonary smooth muscle cells hypoxia increases the expression of both the KCa1.1 α and the β 1 subunits through a HIF-1 α -mediated mechanism (Resnik et al., 2006; Ahn et al., 2012). Conversely in human podocytes hypoxia causes a significant reduction in KCa1.1 channel currents, by an increase of the KCa1.1 β 4 subunit, that causes a shift of the channel activation range toward more depolarized voltages (Zhang et al., 2012a). In alveolar epithelial A549 cells and rat carotid body type I cells KCa1.1 channels appear also sensitive to acute hypoxia (Riesco-Fagundo et al., 2001; Jovanović et al., 2003). In addition hemoxygenase-2 (HO-2) has been found to be part of the KCa1.1 channel complex, and modulate channel activity depending on the oxygen levels (Williams et al., 2004). Hypoxia can also directly inhibit specific KCa1.1 channel splicing isoforms containing a cysteine-serin (CS) motif in its C-terminal segment (McCartney et al., 2005), a feature also displayed by the gBK isoform (Liu et al., 2002). However, a recent study performed in GBM cells has shown that acute hypoxia activates KCa1.1 channels residing in the mitochondrial membrane, while no effect was observed on plasmamembrane KCa1.1 channels (Gu et al., 2014). The effects of chronic hypoxia on the expression or properties of GBM KCa1.1 channels, and whether this modulation has a role in the hypoxia-induced invasive phenotype, remain to be tested. Finally, in intestinal epithelial cells chemical hypoxia modulates the basolateral KCa3.1 activity (Loganathan et al., 2011).

TRPC6 CHANNELS

One of the few cases where a role of ion channels in the hypoxia-induced aggressiveness has been clearly demonstrated is

represented by the Ca^{2+} -permeable transient receptor potential 6 (TRPC6). In GBM cells hypoxia increases Notch1 activation, which induces the expression of TRPC6, resulting in an increased basal intracellular Ca^{2+} concentration and activation of the calcineurin-nuclear factor of activated T-cell (NFAT) pathway (Chigurupati et al., 2010). The hypoxia-induced TRPC6 upregulation is required for the development of the aggressive phenotype as TRPC6 knock-down inhibited glioma growth, invasion, and angiogenesis. Finally, expression of TRPC6 was elevated also in GBM specimens in comparison with normal tissues (Chigurupati et al., 2010).

T-TYPE Ca^{2+} CHANNELS

U87 GBM cells express all three α subunits of T-type Ca^{2+} channels, a class of Ca^{2+} permeable low-voltage activated channels that open in response to small depolarization of the membrane, and whose activity increases the transwell migratory ability of these cells (Zhang et al., 2012b). Notably, hypoxia modulates T-type Ca^{2+} channels in many different cell types. In PC12 cells and adult chromaffin cells, the α 1 subunit mRNA expression and the number of functional T-type Ca^{2+} channels are upregulated by hypoxia (Del Toro et al., 2003; Carabelli et al., 2007). It may thus be possible that a hypoxia-induced upregulation of T-type Ca^{2+} channels in GBM cells participates in enhancing the invasive potential of these cells under hypoxic conditions.

STORE-OPERATED Ca^{2+} CHANNELS

A role in GBM cell migration has also been found for the store-operated Ca^{2+} channels, Ca^{2+} permeable channels whose activity is controlled by the filling level of the intracellular Ca^{2+} stores. In two different primary cultures from GBM biopsies knock-down of the STIM1/ORAI proteins encoding for the store-operated Ca^{2+} channels strongly reduced the serum-induced chemotactic migration through transwell membranes (Motiani et al., 2013). Interestingly, in pulmonary artery smooth muscle cells, acute hypoxia activates the store-operated Ca^{2+} entry (Peng et al., 2013), and in the distal intrapulmonary arteries chronic hypoxia upregulates the expression of STIM1 (Hou et al., 2013). In addition Wilms tumor suppressor 1, a recognized inhibitor of STIM1 expression (Ritchie et al., 2010), has been identified as a target of HIF-1 (Scholz and Kirschner, 2011). All these data point to the suggestion that a hypoxia-induced modulation of the store-operated Ca^{2+} entry may contribute in promoting the invasion of GBM cells.

RADIORESISTANCE

Radiation therapy impairs the growth and survival of tumor cells mainly by causing double strand breaks in the DNA backbone. It is included, as adjuvant therapy, in the surgical and chemotherapeutic treatments of GBM. However, outcomes are poor, and GBM remains an incurable disease with the majority of recurrences and progression within the radiation treatment field (Alexander et al., 2013). Tumor hypoxia represents a severe problem for radiation therapy, as radiosensitivity rapidly decreases when the

O₂ partial pressure is less than 25–30 mmHg. Since in low oxygen the DNA damage speeds up, the radiotherapeutic dose required to achieve the same biologic effect is markedly higher under hypoxia (Gray et al., 1953). Several lines of evidence indicate that hypoxia is involved in the transcriptional modulation of genes that control cell growth and resistance to apoptotic cell death, and the selection of more resistant cell clones, and all these actions may have a major impact on radioresistance (Vaupel et al., 2001; Vaupel, 2004; Amberger-Murphy, 2009; Jensen, 2009). Despite a full course of radiotherapy, up to 90% of GBM relapse in proximity of areas with high HIF-1 expression (Brat and Van Meir, 2004; Rong et al., 2006). In addition the intensity of hypoxia in GBM before radiotherapy is strongly associated with enhanced tumor progression and decreased patient survival (Spence et al., 2008). Recent studies, both in cultured GBM cells and in xerograph models, show that cycling (intermittent) hypoxia induces HIF-1 upregulation, nox4-mediated ROS production and increased radioresistance (Hsieh et al., 2010, 2012).

KCa1.1 CHANNEL

A well-known form of radioresistance is the promotion of tumor cell invasiveness by irradiation (Camphausen et al., 2001; Wild-Bode et al., 2001; Qian et al., 2002; Cordes et al., 2003; Park et al., 2006). Interestingly, it has been found that irradiation of GBM cells raises the intracellular Ca²⁺ concentration and stimulates KCa1.1 channel activity, which result in the activation of CaMKII and enhanced cell migration (Steinle et al., 2011). Notably, both CaMKII activation and the enhanced migration were abolished by the KCa1.1 channel inhibitor paxilline (Steinle et al., 2011). As several studies have shown that in GBM cells and in other tissues KCa1.1 channels are modulated by hypoxia, these results suggest that the hypoxia-mediated modulation of KCa1.1 channels may participate to the hypoxia-induced GBM radioresistance.

RESISTANCE TO APOPTOSIS

It has long been known that hypoxia increases the anti-apoptotic potential of tumor cells by modulating the expression and activity of the molecules involved in the apoptotic pathways (Harris, 2002). In GBM cells held under hypoxia BNIP3, a pro-apoptotic member of the Bcl-2 family, becomes unable to translocate to the cytoplasm and mediate hypoxia-induced cell death, thus favoring cell survival through the loss of apoptotic potential (Burton et al., 2006). Moreover, in GBM cells chronic hypoxia induces Bad phosphorylation and prevents its binding to Bcl-XL, promoting survival of GBM cells (Merighi et al., 2007).

Cl⁻ AND Ca²⁺-ACTIVATED K⁺ CHANNELS

Several ion channels expressed in GBM cells are required for the apoptotic process. An early hallmark in apoptosis is the cellular condensation, termed apoptotic volume decrease (AVD). K⁺ and Cl⁻ effluxes have an essential role in this mechanism as they establish the driving force for cytoplasmic water efflux from the cell. It has been shown that AVD in D54-MG glioma cells is dependent on a DIDS-sensitive chloride conductance (Ernest et al., 2008), and a K⁺ efflux mediated

by Ca²⁺-activated K⁺ channels, KCa3.1 and KCa1.1 (McFerrin et al., 2012). These channels play differential roles in apoptosis: KCa3.1 mediates AVD in response to the activation of the intrinsic pathway, consisting in the mitochondrial disruption by various stressors, whereas KCa1.1 is engaged by the extrinsic pathway, involving the stimulation by extracellular ligands. The involvement of these two channels in different apoptotic pathways is explained by the different temporal Ca²⁺ profiles during induction of apoptosis (McFerrin et al., 2012). We have already mentioned on the evidence of the hypoxia-induced modulation of KCa1.1, KCa3.1, and Cl⁻ channels. If these findings will be confirmed in GBM cells, a role of ion channels in the hypoxia-induced resistance to cell death becomes likely.

TRPV1 AND T-TYPE Ca²⁺ CHANNELS

In U373 glioma cell line, capsaicin stimulation of TRPV1 channels induced a rapid Ca²⁺ influx, DNA fragmentation, externalization of phosphatidylserine, mitochondrial transmembrane potential dissipation, and activation of caspase 3 and p38 MAPK (Amantini et al., 2007). In addition in GBM cell lines T-type Ca²⁺ channels are over-expressed and their activity is correlated with tumor proliferation and progression. Valerie et al. (2013) showed that in glioma cell lines the pharmacological inhibition or siRNA-mediated knock-down of T-type Ca²⁺ channel leads to apoptotic cell death by inhibiting mTOR/Akt pro-survival signaling pathways. We have already discussed the ability of hypoxia to modulate T-type Ca²⁺ channels. As for TRPV1 channel, in pulmonary artery smooth muscle cells, the TRPV1 gene and protein are upregulated by chronic hypoxia (Wang et al., 2008). Both TRPV1 and T-type Ca²⁺ channels may thus potentially be involved in the hypoxia-induced resistance to apoptosis in GBM cells.

CONCLUSIONS

In this perspective we have taken several examples of GBM aggressiveness and showed how they strictly relate to hypoxia and ion channels. Namely, we provided evidence that hypoxia promotes GBM aggressive phenotypes, that several types of ion channel are deeply involved in driving GBM aggressiveness, and that hypoxia heavily modulates these very channels promoting GBM aggressiveness. These lines of evidence are in our view more than a circumstantial coincidence. On the contrary they appear strong indications to put forward the working hypothesis that hypoxia promotes the various forms of GBM aggressiveness through mechanisms involving also ion channels as relevant players. This working hypothesis could be taken as a basis to direct future research.

ACKNOWLEDGMENTS

This project was supported by funds from the MIUR-PRIN (20108WT59Y_004), Ministero della Salute (GR-2009-1580433) and Fondazione Cassa di Risparmio di Perugia (2014/221).

REFERENCES

- Ahn, Y. T., Kim, Y. M., Adams, E., Lyu, S. C., Alvira, C. M., and Cornfield, D. N. (2012). Hypoxia-inducible factor-1 α regulates KCNMB1 expression in human pulmonary artery smooth muscle cells. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 302, L352–L359. doi: 10.1152/ajplung.00302.2011

- Akita, T., and Okada, Y. (2014). Characteristics and roles of the volume-sensitive outwardly rectifying (VSOR) anion channel in the central nervous system. *Neuroscience* 275, 211–231. doi: 10.1016/j.neuroscience.2014.06.015
- Alexander, B. M., Ligon, K. L., and Wen, P. Y. (2013). Enhancing radiation therapy for patients with glioblastoma. *Expert Rev. Anticancer Ther.* 13, 569–581. doi: 10.1586/era.13.44
- Amantini, C., Mosca, M., Nabissi, M., Lucciarini, R., Caprodossi, S., Arcella, A., et al. (2007). Capsaicin-induced apoptosis of glioma cells is mediated by TRPV1 vanilloid receptor and requires p38 MAPK activation. *J. Neurochem.* 102, 977–990. doi: 10.1111/j.1471-4159.2007.04582.x
- Amberger-Murphy, V. (2009). Hypoxia helps glioma to fight therapy. *Curr. Cancer Drug Targets* 9, 381–390. doi: 10.2174/156800909788166637
- Bordey, A., Sontheimer, H., and Trouslard, J. (2000). Muscarinic activation of BK channels induces membrane oscillations in glioma cells and leads to inhibition of cell migration. *J. Membr. Biol.* 176, 31–40. doi: 10.1007/s002320001073
- Brat, D. J., and Van Meir, E. G. (2004). Vaso-occlusive and prothrombotic mechanisms associated with tumor hypoxia, necrosis and accelerated growth in glioblastoma. *Lab. Invest.* 84, 397–405. doi: 10.1038/labinvest.3700070
- Burton, T. R., Hensonm, E. S., Bajajalm, P., Eisenstatm, D. D., and Gibson, S. B. (2006). The pro-cell death Bcl-2 family member, BNIP3, is localized to the nucleus of human glial cells: implications for glioblastoma multiforme tumor cell survival under hypoxia. *Int. J. Cancer.* 118, 1660–1669. doi: 10.1002/ijc.21547
- Camphausen, K., Moses, M. A., Beecken, W. D., Khan, M. K., Folkman, J., and O'Reilly, M. S. (2001). Radiation therapy to a primary tumor accelerates metastatic growth in mice. *Cancer Res.* 61, 2207–2211.
- Carabelli, V., Marcantoni, A., Comunanza, V., and Carbone, E. (2007). Fast exocytosis mediated by T- and L-type channels in chromaffin cells: distinct voltage-dependence but similar Ca^{2+} -dependence. *Eur. Biophys. J.* 36, 753–762. doi: 10.1007/s00249-007-0138-2
- Catacuzzeno, L., Aiello, F., Fioretti, B., Sforna, L., Castigli, E., Ruggieri, P., et al. (2011). Serum-activated K and Cl currents underlay U87-MG glioblastoma cell migration. *J. Cell. Physiol.* 226, 1926–1933. doi: 10.1002/jcp.22523
- Catacuzzeno, L., Fioretti, B., and Franciolini, F. (2012). Expression and role of the intermediate-conductance calcium-activated potassium channel $KCa_{3.1}$ in glioblastoma. *J. Signal Transduct.* 2012:421564. doi: 10.1155/2012/421564
- Catacuzzeno, L., Michelucci, A., Sforna, L., Aiello, F., Sciaccaluga, M., Fioretti, B., et al. (2014). Identification of key signaling molecules involved in the activation of the swelling-activated chloride current in human glioblastoma cells. *J. Membr. Biol.* 247, 45–55. doi: 10.1007/s00232-013-9609-9
- Chigurupati, S., Venkataraman, R., Barrera, D., Naganathan, A., Madan, M., Paul, L., et al. (2010). Receptor channel TRPC6 is a key mediator of Notch-driven glioblastoma growth and invasiveness. *Cancer Res.* 70, 418–427. doi: 10.1158/0008-5472.can-09-2654
- Claes, A., Idema, A. J., and Wesseling, P. (2007). Diffuse glioma growth: a guerilla war. *Acta Neuropathol.* 114, 443–458. doi: 10.1007/s00401-007-0293-7
- Comerford, K. M., Wallace, T. J., Karhausen, J., Louis, N. A., Montalto, M. C., and Colgan, S. P. (2002). Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. *Cancer Res.* 62, 3387–3394.
- Cordes, N., Hansmeier, B., Beinke, C., Meineke, V., and van Beuningen, D. (2003). Irradiation differentially affects substratum-dependent survival, adhesion and invasion of glioblastoma cell lines. *Br. J. Cancer.* 89, 2122–2132.
- Cuddapah, V. A., and Sontheimer, H. (2011). Ion channels and transporters [corrected] in cancer. 2. Ion channels and the control of cancer cell migration. *Am. J. Physiol. Cell Physiol.* 301, C541–C549. doi: 10.1152/ajpcell.00102.2011
- Del Toro, R., Levitsky, K. L., López-Barneo, J., and Chiara, M. D. (2003). Induction of T-type calcium channel gene expression by chronic hypoxia. *J. Biol. Chem.* 278, 22316–22324. doi: 10.1074/jbc.m212576200
- Deshane, J., Garner, C. C., and Sontheimer, H. (2003). Chlorotoxin inhibits glioma cell invasion via matrix metalloproteinase-2. *J. Biol. Chem.* 278, 4135–4144. doi: 10.1074/jbc.m205662200
- Dunn, C. A., and Lampe, P. D. (2014). Injury-triggered Akt phosphorylation of $Cx43$: a ZO-1-driven molecular switch that regulates gap junction size. *J. Cell Sci.* 127, 455–464. doi: 10.1242/jcs.142497
- Ernest, N. J., Habela, C. W., and Sontheimer, H. (2008). Cytoplasmic condensation is both necessary and sufficient to induce apoptotic cell death. *J. Cell Sci.* 121, 290–297. doi: 10.1242/jcs.017343
- Ernest, N. J., Weaver, A. K., Van Duyn, L. B., and Sontheimer, H. W. (2005). Relative contribution of chloride channels and transporters to regulatory volume decrease in human glioma cells. *Am. J. Physiol. Cell Physiol.* 288, C1451–C1460. doi: 10.1152/ajpcell.00503.2004
- Esencay, M., Sarfraz, Y., and Zagzag, D. (2013). CXCR7 is induced by hypoxia and mediates glioma cell migration towards SDF-1 α . *BMC Cancer* 13:347. doi: 10.1186/1471-2407-13-347
- Fry, T., Evans, J. H., and Sanderson, M. J. (2001). Propagation of intercellular calcium waves in C6 glioma cells transfected with connexins 43 or 32. *Microsc. Res. Tech.* 52, 289–300. doi: 10.1002/1097-0029(20010201)52:3<289::aid-jemt1014>3.0.co;2-0
- Fujimura, A., Michiue, H., Cheng, Y., Uneda, A., Tani, Y., Nishiki, T., et al. (2013). Cyclin G2 promotes hypoxia-driven local invasion of glioblastoma by orchestrating cytoskeletal dynamics. *Neoplasia* 15, 1272–1281. doi: 10.1593/neo.131440
- Gielen, P. R., Aftab, Q., Ma, N., Chen, V. C., Hong, X., Lozinsky, S., et al. (2013). Connexin43 confers Temozolomide resistance in human glioma cells by modulating the mitochondrial apoptosis pathway. *Neuropharmacology* 75, 539–548. doi: 10.1016/j.neuropharm.2013.05.002
- Goldberg, G. S., Lampe, P. D., and Nicholson, B. J. (1999). Selective transfer of endogenous metabolites through gap junctions composed of different connexins. *Nat. Cell Biol.* 1, 457–459.
- Goodenough, D. A., and Paul, D. L. (2003). Beyond the gap: functions of unpaired connexon channels. *Nat. Rev. Mol. Cell Biol.* 4, 285–294. doi: 10.1038/nrm1072
- Gray, L. H., Conger, A. D., Ebert, M., Hornsey, S., and Scott, O. C. (1953). The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br. J. Radiol.* 26, 638–648. doi: 10.1259/0007-1285-26-312-638
- Grayson, W. L., Zhao, F., Bunnell, B., and Ma, T. (2007). Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochem. Biophys. Res. Commun.* 358, 948–953. doi: 10.1016/j.bbrc.2007.05.054
- Gu, X. Q., Pamerter, M. E., Siemen, D., Sun, X., and Haddad, G. G. (2014). Mitochondrial but not plasmalemmal BK channels are hypoxia-sensitive in human glioma. *Glia* 62, 504–513. doi: 10.1002/glia.22620
- Haar, C. P., Hebbbar, P., Wallace, G. C. 4th, Das, A., Vandergrift, W. A. 3rd, Smith, J. A., et al. (2012). Drug resistance in glioblastoma: a mini review. *Neurochem. Res.* 37, 1192–1200. doi: 10.1007/s11064-011-0701-1
- Harris, A. L. (2002). Hypoxia—a key regulatory factor in tumour growth. *Nat. Rev. Cancer.* 2, 38–47. doi: 10.1038/nrc704
- Holland, E. C. (2001). Gliomagenesis: genetic alterations and mouse models. *Nat. Rev. Genet.* 2, 120–129
- Hou, X., Chen, J., Luo, Y., Liu, F., Xu, G., and Gao, Y. (2013). Silencing of STIM1 attenuates hypoxia-induced PSMCs proliferation via inhibition of the SOC/ Ca^{2+} /NFAT pathway. *Respir. Res.* 14:2. doi: 10.1186/1465-9921-14-2
- Hsieh, C. H., Lee, C. H., Liang, J. A., Yu, C. Y., and Shyu, W. C. (2010). Cycling hypoxia increases U87 glioma cell radioresistance via ROS induced higher and long-term HIF-1 signal transduction activity. *Oncol. Rep.* 24, 1629–1636. doi: 10.3892/or.00001027
- Hsieh, C. H., Wu, C. P., Lee, H. T., Liang, J. A., Yu, C. Y., and Lin, Y. J. (2012). NADPH oxidase subunit 4 mediates cycling hypoxia-promoted radiation resistance in glioblastoma multiforme. *Free Radic. Biol. Med.* 53, 649–658. doi: 10.1016/j.freeradbiomed.2012.06.009
- Huang, R. P., Fan, Y., Hossain, M. Z., Peng, A., Zeng, Z. L., and Boynton, A. L. (1998). Reversion of the neoplastic phenotype of human glioblastoma cells by connexin 43 (cx43). *Cancer Res.* 58, 5089–5096.
- Jensen, R. L. (2009). Brain tumor hypoxia: tumorigenesis, angiogenesis, imaging, pseudoprogression and as a therapeutic target. *J. Neurooncol.* 92, 317–335. doi: 10.1007/s11060-009-9827-2
- Jovanović, S., Crawford, R. M., Ranki, H. J., and Jovanović, A. (2003). Large conductance Ca^{2+} -activated K^{+} channels sense acute changes in oxygen tension in alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 28, 363–372. doi: 10.1165/rcmb.2002-0101oc
- Kang, M. K., and Kang, S. K. (2008). Pharmacologic blockade of chloride channel synergistically enhances apoptosis of chemotherapeutic drug-resistant cancer stem cells. *Biochem. Biophys. Res. Commun.* 373, 539–544. doi: 10.1016/j.bbrc.2008.06.070
- Lefranc, F., Rynkowski, M., DeWitte, O., and Kiss, R. (2009). Present and potential future adjuvant issues in high-grade astrocytic glioma treatment. *Adv. Tech. Stand. Neurosurg.* 34, 3–35. doi: 10.1007/978-3-211-78741-0_1
- Li, W. E., and Nagy, J. I. (2000). Connexin43 phosphorylation state and intercellular communication in cultured astrocytes following hypoxia and protein

- phosphatase inhibition. *Eur. J. Neurosci.* 12, 2644–2650. doi: 10.1046/j.1460-9568.2000.00162.x
- Liu, X., Chang, Y., Reinhart, P. H., Sontheimer, H., and Chang, Y. (2002). Cloning and characterization of glioma BK, a novel BK channel isoform highly expressed in human glioma cells. *J. Neurosci.* 22, 1840–1849.
- Loganathan, A., Linley, J. E., Rajput, I., Hunter, M., Lodge, J. P., and Sandle, G. I. (2011). Basolateral potassium (IKCa) channel inhibition prevents increased colonic permeability induced by chemical hypoxia. *Am. J. Physiol. Gastrointest. Liver Physiol.* 300, G146–G153. doi: 10.1152/ajpgi.00472.2009
- López-Barneo, J., López-López, J. R., Ureña, J., and González, C. (1988). Chemotransduction in the carotid body: K⁺ current modulated by PO₂ in type I chemoreceptor cells. *Science* 241, 580–582. doi: 10.1126/science.2456613
- McCartney, C. E., McClafferty, H., Huibant, J. M., Rowan, E. G., Shipston, M. J., and Rowe, I. C. (2005). A cysteine-rich motif confers hypoxia sensitivity to mammalian large conductance voltage- and Ca-activated K (BK) channel alpha-subunits. *Proc. Natl. Acad. Sci. U S A* 102, 17870–17876. doi: 10.1073/pnas.0505270102
- McFerrin, M. B., and Sontheimer, H. (2006). A role for ion channels in glioma cell invasion. *Neuron Glia Biol.* 2, 39–49. doi: 10.1017/s1740925x06000044
- McFerrin, M. B., Turner, K. L., Cuddapah, V. A., and Sontheimer, H. (2012). Differential role of IK and BK potassium channels as mediators of intrinsic and extrinsic apoptotic cell death. *Am. J. Physiol. Cell Physiol.* 303, C1070–C1078. doi: 10.1152/ajpcell.00040.2012
- Méndez, O., Zavadil, J., Esencay, M., Lukyanov, Y., Santovasi, D., Wang, S. C., et al. (2010). Knock down of HIF-1alpha in glioma cells reduces migration in vitro and invasion in vivo and impairs their ability to form tumor spheres. *Mol. Cancer* 9:133. doi: 10.1186/1476-4598-9-133
- Merighi, S., Benini, A., Mirandola, P., Gessi, S., Varani, K., Leung, E., et al. (2007). Hypoxia inhibits paclitaxel-induced apoptosis through adenosine-mediated phosphorylation of bad in glioblastoma cells. *Mol. Pharmacol.* 72, 162–172. doi: 10.1124/mol.106.031849
- Motiani, R. K., Hyzinski-García, M. C., Zhang, X., Henkel, M. M., Abdullaev, I. F., Kuo, Y. H., et al. (2013). STIM1 and Orai1 mediate CRAC channel activity and are essential for human glioblastoma invasion. *Pflugers Arch.* 465, 1249–1260. doi: 10.1007/s00424-013-1254-8
- Naus, C. C., and Laird, D. W. (2010). Implications and challenges of connexin connections to cancer. *Nat. Rev. Cancer* 10, 435–441. doi: 10.1038/nrc2841
- Oliver, L., Olivier, C., Marhuenda, F. B., Campone, M., and Vallette, F. M. (2009). Hypoxia and the malignant glioma microenvironment: regulation and implications for therapy. *Curr. Mol. Pharmacol.* 2, 263–284. doi: 10.2174/1874467210902030263
- Olsen, M. L., Schade, S., Lyons, S. A., Amaral, M. D., and Sontheimer, H. (2003). Expression of voltage-gated chloride channels in human glioma cells. *J. Neurosci.* 23, 5572–5582.
- Park, C. M., Park, M. J., Kwak, H. J., Lee, H. C., Kim, M. S., Lee, S. H., et al. (2006). Ionizing radiation enhances matrix metalloproteinase-2 secretion and invasion of glioma cells through Src/epidermal growth factor receptor-mediated p38/Akt and phosphatidylinositol 3-kinase/Akt signaling pathways. *Cancer Res.* 66, 8511–8519. doi: 10.1158/0008-5472.can-05-4340
- Patel, A. J., Lauritzen, I., Lazdunski, M., and Honoré, E. (1998). Disruption of mitochondrial respiration inhibits volume-regulated anion channels and provokes neuronal cell swelling. *J. Neurosci.* 18, 3117–3123.
- Peng, G., Ran, P., Lu, W., Zhong, N., and Wang, J. (2013). Acute hypoxia activates store-operated Ca(2+) entry and increases intracellular Ca(2+) concentration in rat distal pulmonary venous smooth muscle cells. *J. Thorac. Dis.* 5, 605–612. doi: 10.3978/j.issn.2072-1439.2013.08.68
- Qian, L. W., Mizumoto, K., Urashima, T., Nagai, E., Maehara, N., Sato, N., et al. (2002). Radiation-induced increase in invasive potential of human pancreatic cancer cells and its blockade by a matrix metalloproteinase inhibitor, CGS27023. *Clin. Cancer Res.* 8, 1223–1227.
- Resnik, E., Herron, J., Fu, R., Ivy, D. D., and Cornfield, D. N. (2006). Oxygen tension modulates the expression of pulmonary vascular BKCa channel alpha- and beta-subunits. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 290, L761–L768. doi: 10.1152/ajplung.00283.2005
- Riesco-Fagundo, A. M., Pérez-García, M. T., González, C., and López-López, J. R. (2001). O₂ modulates large-conductance Ca(2+)-dependent K(+) channels of rat chemoreceptor cells by a membrane restricted and CO-sensitive mechanism. *Circ. Res.* 89, 430–436. doi: 10.1161/hh1701.095632
- Ritchie, M. F., Yue, C., Zhou, Y., Houghton, P. J., and Soboloff, J. (2010). Wilms tumor suppressor 1 (WT1) and early growth response 1 (EGR1) are regulators of STIM1 expression. *J. Biol. Chem.* 285, 10591–10596. doi: 10.1074/jbc.m109.083493
- Rong, Y., Durden, D. L., Van Meir, E. G., and Brat, D. J. (2006). “Pseudopalisading” necrosis in glioblastoma: a familiar morphologic feature that links vascular pathology, hypoxia and angiogenesis. *J. Neuropathol. Exp. Neurol.* 65, 529–539. doi: 10.1097/00005072-200606000-00001
- Santoni, G., Santoni, M., and Nabissi, M. (2012). Functional role of T-type calcium channels in tumour growth and progression: prospective in cancer therapy. *Br. J. Pharmacol.* 166, 1244–1246. doi: 10.1111/j.1476-5381.2012.01908.x
- Scholz, H., and Kirschner, K. M. (2011). Oxygen-Dependent gene expression in development and cancer: lessons learned from the wilms’ tumor gene, WT1. *Front. Mol. Neurosci.* 4:4. doi: 10.3389/fnmol.2011.00004
- Sciacaluga, M., Fioretti, B., Catacuzzeno, L., Pagani, F., Bertolini, C., Rosito, M., et al. (2010). CXCL12-induced glioblastoma cell migration requires intermediate conductance Ca²⁺-activated K⁺ channel activity. *Am. J. Physiol. Cell Physiol.* 299, C175–C184. doi: 10.1152/ajpcell.00344.2009
- Severino, A., Narducci, M. L., Piccinino, D., Pazzano, V., Giglio, A. F., Biasucci, L. M., et al. (2012). Reversible atrial gap junction remodeling during hypoxia/reoxygenation and ischemia: a possible arrhythmogenic substrate for atrial fibrillation. *Gen. Physiol. Biophys.* 31, 439–448. doi: 10.4149/gpb_2012_047
- Shen, S. H., Kwan, A. L., Chen, Y. Y., and Wang, Z. X. (2013). Effect of silencing HIF-1α on proliferation, invasion and migration of glioblastoma U87 cells. *Neurol. Sci.* 34, 365–371. doi: 10.1007/s10072-012-1010-4
- Shimoda, L. A., and Polak, J. (2011). Hypoxia. 4. Hypoxia and ion channel function. *Am. J. Physiol. Cell Physiol.* 300, C951–C967. doi: 10.1152/ajpcell.00512.2010
- Simon, A. M., and Goodenough, D. A. (1998). Diverse functions of vertebrate gap junctions. *Trends Cell Biol.* 8, 477–483. doi: 10.1016/s0962-8924(98)01372-5
- Sontheimer, H. (2004). Ion channels and amino acid transporters support the growth and invasion of primary brain tumors. *Mol. Neurobiol.* 29, 61–71. doi: 10.1385/mn:29:1:61
- Sontheimer, H. (2008). An unexpected role for ion channels in brain tumor metastasis. *Exp. Biol. Med. (Maywood)* 233, 779–791. doi: 10.3181/0711-mr-308
- Spence, A. M., Muzi, M., Swanson, K. R., O’Sullivan, F., Rockhill, J. K., Rajendran, J. G., et al. (2008). Regional hypoxia in glioblastoma multiforme quantified with [18F]fluoromisonidazole positron emission tomography before radiotherapy: correlation with time to progression and survival. *Clin. Cancer Res.* 14, 2623–2630. doi: 10.1158/1078-0432.ccr-07-4995
- Steinle, M., Palme, D., Misovic, M., Rudner, J., Dittmann, K., Lukowski, R., et al. (2011). Ionizing radiation induces migration of glioblastoma cells by activating BK K(+) channels. *Radiother. Oncol.* 101, 122–126. doi: 10.1016/j.radonc.2011.05.069
- Su, J., Xu, Y., Zhou, L., Yu, H. M., Kang, J. S., Liu, N., et al. (2013). Suppression of chloride channel 3 expression facilitates sensitivity of human glioma U251 cells to cisplatin through concomitant inhibition of Akt and autophagy. *Anat. Rec. (Hoboken)* 296, 595–603. doi: 10.1002/ar.22665
- Sun, H., Xia, Y., Paudel, O., Yang, X. R., and Sham, J. S. (2012). Chronic hypoxia-induced upregulation of Ca²⁺-activated Cl⁻ channel in pulmonary arterial myocytes: a mechanism contributing to enhanced vasoreactivity. *J. Physiol.* 590, 3507–3521. doi: 10.1113/jphysiol.2012.232520
- Turner, K. L., and Sontheimer, H. (2014). Cl⁻ and K⁺ channels and their role in primary brain tumour biology. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369:20130095. doi: 10.1098/rstb.2013.0095
- Valerie, N. C., Dziegielewska, B., Hosing, A. S., Augustin, E., Gray, L. S., Brautigan, D. L., et al. (2013). Inhibition of T-type calcium channels disrupts Akt signaling and promotes apoptosis in glioblastoma cells. *Biochem. Pharmacol.* 85, 888–897. doi: 10.1016/j.bcp.2012.12.017
- Vaupel, P. (2004). Tumor microenvironmental physiology and its implications for radiation oncology. *Semin. Radiat. Oncol.* 14, 198–206. doi: 10.1016/j.semradonc.2004.04.008
- Vaupel, P., Thews, O., and Hoekel, M. (2001). Treatment resistance of solid tumors: role of hypoxia and anemia. *Med. Oncol.* 18, 243–259. doi: 10.1385/mo:18:4:243
- Wang, Y. X., Wang, J., Wang, C., Liu, J., Shi, L. P., Xu, M., et al. (2008). Functional expression of transient receptor potential vanilloid-related channels in

- chronically hypoxic human pulmonary arterial smooth muscle cells. *J. Membr. Biol.* 223, 151–159. doi: 10.1007/s00232-008-9121-9
- Wild-Bode, C., Weller, M., Rimmer, A., Dichgans, J., and Wick, W. (2001). Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma. *Cancer Res.* 61, 2744–2750.
- Williams, S. E., Wootton, P., Mason, H. S., Bould, J., Iles, D. E., Riccardi, D., et al. (2004). Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science* 306, 2093–2097. doi: 10.1126/science.1105010
- Wongergem, R., and Bartley, J. W. (2009). Menthol increases human glioblastoma intracellular Ca^{2+} , BK channel activity and cell migration. *J. Biomed. Sci.* 16:90. doi: 10.1186/1423-0127-16-90
- Wu, X., Huang, W., Luo, G., and Alain, L. A. (2013). Hypoxia induces connexin 43 dysregulation by modulating matrix metalloproteinases via MAPK signaling. *Mol. Cell. Biochem.* 384, 155–162. doi: 10.1007/s11010-013-1793-5
- Yang, L., Lin, C., Wang, L., Guo, H., and Wang, X. (2012). Hypoxia and hypoxia-inducible factors in glioblastoma multiforme progression and therapeutic implications. *Exp. Cell Res.* 318, 2417–2426. doi: 10.1016/j.yexcr.2012.07.017
- Zhang, Y., Liu, Q., Wang, F., Ling, E. A., Liu, S., Wang, L., et al. (2013). Melatonin antagonizes hypoxia-mediated glioblastoma cell migration and invasion via inhibition of HIF-1 α . *J. Pineal Res.* 55, 121–130. doi: 10.1111/jpi.12052
- Zhang, R., Sun, H., Liao, C., Yang, H., Zhao, B., Tian, J., et al. (2012a). Chronic hypoxia in cultured human podocytes inhibits BKCa channels by upregulating its $\beta 4$ -subunit. *Biochem. Biophys. Res. Commun.* 420, 505–510. doi: 10.1016/j.bbrc.2012.03.021
- Zhang, Y., Zhang, J., Jiang, D., Zhang, D., Qian, Z., Liu, C., et al. (2012b). Inhibition of T-type Ca^{2+} channels by endostatin attenuates human glioblastoma cell proliferation and migration. *Br. J. Pharmacol.* 166, 1247–1260. doi: 10.1111/j.1476-5381.2012.01852.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 November 2014; accepted: 24 December 2014; published online: 15 January 2015.

Citation: Sforna L, Cenciarini M, Belia S, D'Adamo MC, Pessia M, Franciolini F and Catacuzzeno L (2015) The role of ion channels in the hypoxia-induced aggressiveness of glioblastoma. *Front. Cell. Neurosci.* 8:467. doi: 10.3389/fncel.2014.00467

This article was submitted to the journal *Frontiers in Cellular Neuroscience*.

Copyright © 2015 Sforna, Cenciarini, Belia, D'Adamo, Pessia, Franciolini and Catacuzzeno. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution and reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.