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Original Research Article

BK channels blockage inhibits hypoxia-induced migration and chemoresistance to cisplatin in human glioblastoma cells

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Running head: BK channels in human glioblastoma cells

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ABSTRACT

Glioblastoma (GBM) cells express large-conductance, calcium-activated potassium (BK) channels, whose activity is important for several critical aspects of the tumor, such as migration/invasion and cell death. GBMs are also characterized by a heavy hypoxic microenvironment that exacerbates tumor aggressiveness. Since hypoxia modulates the activity of BK channels in many tissues, we hypothesized that a hypoxia-induced modulation of these channels may contribute to the hypoxia-induced GBM aggressiveness. In U87-MG cells, hypoxia induced a functional upregulation of BK channel activity, without interfering with their plasma membrane expression. Wound healing and transwell migration assays showed that hypoxia increased the migratory ability of U87-MG cells, an effect that could be prevented by BK channel inhibition. Toxicological experiments showed that hypoxia was able to induce chemoresistance to cisplatin in U87-MG cells and that the inhibition of BK channels prevented the hypoxia-induced chemoresistance. Clonogenic assays showed that BK channels are also used to increase the clonogenic ability of U87-MG GBM cells in presence, but not in absence, of cisplatin. BK channels were also found to be essential for the hypoxia-induced de-differentiation of GBM cells. Finally, using immunohistochemical analysis, we highlighted the presence of BK channels in hypoxic areas of human GBM tissues, suggesting that our findings may have physiopathological relevance in vivo. In conclusion, our data show that BK channels promote several aspects of the aggressive potential of GBM cells induced by hypoxia, such as migration and chemoresistance to cisplatin, suggesting it as a potential therapeutic target in the treatment of GBM.

INTRODUCTION

Glioma are glial tumors of the central nervous system making up 35-50% of all intracranial tumors in adult age. Grade IV GBM is the most aggressive form of glioma with the highest incidence and a median survival of approximately 20 months in patients who undergo total surgical resection, radio- and chemotherapy (Holland, 2001). Among the most commonly used chemotherapeutic agents in GBM treatment there are temozolomide, a precursor of the DNA methylating agent monomethyl-triazenoimidazole carbaxamide, and cisplatin, especially useful in combination with temozolomide (Brandes *et al.*, 2004; Zustovich *et al.*, 2009). The failure of current therapies is for a major part due to the high capacity of GBM cells to migrate and invade the surrounding brain parenchyma, and to resist to conventional chemotherapeutic treatments (Maher *et al.*, 2001). GBM are characterized by extensive infiltration of tumor cells in healthy brain parenchyma. Several mechanisms and factors are relevant to migration and infiltration of GBM cells, the most critical being an intracellular integration system of signals originating from membrane receptors (growth

factors, integrins, etc.) and other stimulatory factors in the tumor microenvironment, and a cellular actin cytoskeleton-based locomotor apparatus that physically moves GBM cells. However, cell movement also requires the participation of K and Cl channels to allow cells change their volume, absolute requirement for locomotion. As for drug resistance, standard chemotherapies are designed to inhibit cancer cell growth by activating cell death pathways that lead to apoptosis or autophagy. In preclinical studies, agents targeting these pathways have been shown to sensitize cancer cells to chemotherapy. However, as the disease progresses, cancer cells acquire additional genetic and epigenetic alterations that deregulate these signaling pathways, and in turn lead to chemoresistance. On this ground, there is an essential need to better understand the molecular mechanisms of cell migration and chemoresistance through which GBM cells are able to exert their aggressive phenotype in order to improve patients outcome.

As most solid tumors, GBM is characterized by extensive hypoxic areas strongly correlated with tumor malignancy. Low oxygen levels have indeed been shown to promote both invasiveness and chemoresistance (Oliver *et al.*, 2009). In human pulmonary adenocarcinoma A549 cells hypoxia facilitates cell migration through upregulation of HIF-1-dependent gene expression, with resulting redistribution of E-cadherin and b-catenin and reorganization of actin cytoskeleton (Shen *et al.*, 2008). In GBM cells (SNB75 and U87) hypoxia enhances migration and invasion by promoting a mesenchymal shift mediated by the HIF1α–ZEB1 axis (Joseph *et al.*, 2015). Distinct pathways from HIFs have been also described leading to hypoxia-induced cell cancer (breast) migration, as the PERK/TF4/LAMP-3 (Nagelkerke *et al.*, 2013), or upregulation of the chemokine receptor CXCR4 (Cronin, Wang and Redmond, 2010). Lack of oxygen is also taken as a concurring cause of chemoresistance in several cancer types, in that it induces genetic instability, increases MDR transporter P-glycoprotein, deregulates signaling pathways controlling cell proliferation and apoptosis. Lack of oxygen also impairs effective action of anticancer drugs. A number of other cellular processes or pathways regulated by hypoxia may however play relevant roles in hypoxia-induced drug resistance.

The large conductance, Ca^{2+}-activated K (BK) channel, highly expressed in GBM tissues (Sontheimer, 2008; Turner and Sontheimer, 2014), has been thoroughly characterized from a biophysical, structural and functional standpoint. The BK channel structure comprises a pore-forming α subunit, which is encoded by the *slo1* gene (also called KCNMA1), and can be differently processed into many splicing isoforms. In GBM, a particular isoform of BK channels called gBK (glioma BK) is overexpressed compared to normal astrocytes (Liu *et al.*, 2002). The α subunit of BK channels can be regulated by β subunits (β1-4), which are expressed in a tissue-specific manner with the β4 being the most abundant in the brain (Torres, Granados and Latorre, 2014) and in GBM cells (Bednarczyk *et al.*, 2013). BK channels are often coordinated with Cl channels, and together they influence cell migration, proliferation and death by orchestrating ions and water movements,

fundamental for membrane potential and cell volume changes (Cuddapah and Sontheimer, 2011). Specifically, BK channels have been found to be involved in GBM cell migration and invasiveness (Catacuzzeno *et al.*, 2015). For example, ionizing radiations are able to induce the migration of GBM cells in a Ca^{2+}-dependent manner, and the role for BK channels in this process is primary (Steinle *et al.*, 2011). Further, menthol induces GBM cell migration by its influence on BK channels (Weaver, Bomben and Sontheimer, 2006). In addition BK channels have also been found to have a fundamental role in the apoptotic volume decrease of GBM cells induced by TRAIL (McFerrin *et al.*, 2012). All these data highlight the involvement of BK channels in the migratory and invasive properties of GBM cells and in chemoresistance to therapeutic agents.

It can be postulated that hypoxia promotes cell migration and chemoresistance by modulating the BK channel activity or expression (Sforna *et al.*, 2014). Although this type of modulation has not been shown in GBM models, it has been reported in other cell types (Ahn *et al.*, 2012; R. Zhang *et al.*, 2012). Based on these findings we set out to investigate whether BK channels are involved in the hypoxia-induced aggressiveness of GBM cells, in particular their contribution to the chemoresistance and the migratory processes.

MATERIALS AND METHODS

***Cell culture*.** U87-MG glioblastoma multiforme cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and was grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich St. Louis, MO, USA), 100 IU/ml penicillin G, 100 µg/ml streptomycin, 1% L-glutamine, 1% nonessential amino acids and 1 mM sodium pyruvate at 37°C in a 5% CO2-humidified atmosphere. The medium was changed twice a week, and the cells were subcultured when confluent. Glioblastoma primary cultures (WHO grade IV) were obtained in a previous work by our group from tumor specimens of patients (Calogero *et al.*, 2004). For hypoxia experiments, cells were incubated and treated in a GasPak system (BD Biosciences) and flushed with 95% N_2 and 5% CO_2 at 37°C.

***Electrophysiology*.** The whole-cell dialyzed configuration was used for electrophysiological recordings from Glioblastoma cells (Fioretti *et al.*, 2006; Sforna *et al.*, 2017). Currents and voltages were amplified with a HEKA EPC-10 amplifier (List Medical, Darmstadt, Germany), and analyzed with the Patch-Master package (version 2X69, HEKA ELEKTRONIK) and Microcal Origin 8.0 software. For on-line data collection, macroscopic currents were filtered at 3 kHz and sampled at 200 µs/point. The external solution contained (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, MOPS 5, glucose 10, (pH 7.4). Octanol (1 mM) was added to the external bathing solution to block gap-

junctions (Eskandari *et al.*, 2002; Catacuzzeno *et al.*, 2011). TRAM-34 (3 µM) was added to block Ca^{2+}-activated large- and intermediate-conductance K channels, expressed in these cells. The internal solution contained: KCl 155, EGTA-K 1, MOPS 5, $MgCl_2$ 1 (pH 7.2). Access resistances ranging between 4 and 8 MΩ were actively compensated to *ca.* 50%. All chemicals used were of analytical grade. Experiments were carried out at room temperature (18–22°C). Data are presented as mean ± SE. Statistical differences between experimental groups were verified by using the *t*-test, and considering a level of significance (*p*) of 0.05.

RNA extraction and Real Time PCR. Total RNA was isolated from U87-MG using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. To extract RNA, cultured cells were grown in a 60-mm dish to 60–80% confluency and lysed. mRNA concentration was quantified using a DU 800 Spectrophotometer (Beckman Coulter). One µg of mRNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, CA, USA) according to the manufacturer's instructions. Gene expression was quantified by real-time PCR using the 7900HT Fast Real-Time PCR System and Power SYBR Green PCR Master Mix (Applied Biosystem, Warrington, UK) according to the manufacturer's instructions and analyzed. Each experiment was performed in triplicate and is expressed as mean±SD. Experiments were independently repeated three times. Gene expression levels were quantified from real-time PCR data by the comparative threshold cycle (CT) method using the housekeeping gene 18S as an internal reference. The following gene-specific primers were used: human KCNMA1 (QuantiTect Primer Assay, QT00024157, Qiagen, Hilden, Germany); human VEGF FW 5'-CTACCTCCACCATGCCAAGT-3', RV 5'-CCACTTCGTGATTCTGC-3'.

Western blot. Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors, 1mM PMSF, 1mM DTT and 0.5 mM sodium orthovanadate (Sigma-Aldrich). Protein concentration was determined by the Bradford assay and 40 µg of proteins per sample were resolved on an 8% SDS-PAGE gel and blotted onto a PVDF membrane (Amersham HyBond-P GE Healthcare, UK). After blocking at RT in 5% dry-milk in TBS containing 0.1% Tween-20 for 1 hour, membranes were incubated overnight at 4°C with rabbit polyclonal anti-BK channel antibody (Alomone Labs, APC-151, dilution 1:500,) and mouse monoclonal anti-β-actin (Santa Cruz, sc-47778, dilution 1:2000) antibody was used for normalization. Membranes were, then, incubated with anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibodies (dilution 1:10,000, GE Healthcare Bio-Sciences). Immunocomplexes were detected by ECL Western Blotting detection system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Densitometric analysis was performed with Image Studio software (LI-COR Biosciences).

Cytometric analysis. Biparametric analysis was performed by BK/AlexaFluor488 indirect staining. Briefly, U87-MG cells were collected, washed and resuspended in PBS, 2% FBS (106 cells/100 µl).

Samples were incubated for 30 minutes at 4°C with 10 µl of anti-BK (APC-151, Alomone Labs, Jerusalem, Israel), washed and resuspended again in 100 µl of PBS, 2% FBS. AlexaFluor 488-conjugated mouse anti-rabbit antibody (final dilution 1:250, A11034, Life Technologies) was then added and the samples were incubated for additional 30 minutes at 4°C. At the end of incubation, cells were washed again, resuspended in PBS, 2% FBS and the samples were acquired on a FACs ARIA II instrument using FACs DiVa software (v.6.1.1, both by Becton Dickinson). At least 20,000 events were recorded and analyzed using Flowing software (v2.5.1, Turku Centre for Biotechnology, Finland).

Wound healing assay. $4x10^5$ cells were seeded in 60mm tissue culture-treated dishes and grown until confluence. A scratch was made across the cell layer using a sterile p1000 pipette tip and plates were immediately photographed. The medium was replaced and cells were treated with 2 µM paxilline either in hypoxia or normoxia. Plates were re-photographed after 24h. TScratch software(Gebäck et al., 2009) was used to analyze the images and results were indicated as percentage of closure of the wound. All experiments were performed at least three times in triplicates.

Transwell migration assay. Cells were harvested, disaggregated and resuspended in DMEM with 0.2% FBS and $5x10^4$ cells were seeded in 24-well cell culture inserts with 8 µm pore size membrane (Boyden chambers, BD Biosciences). DMEM supplemented with 10% FBS was added to the lower chamber as chemo-attractant. BK channel blocker paxilline was added to DMEM at a concentration of 2 µM and applied in both the upper and lower part of the chamber. Cells were incubated at 37°C for 18 hours, either in normoxia or hypoxia. After this time, cells were fixed with 100% methanol and non migrated cells were removed from the inner side of the insert with a cotton swab. Migrated cells, which had the ability to push themselves through the 8 µm pores, were gently rinsed with PBS, stained with 0.25% crystal violet (Sigma-Aldrich) for 15 minutes, rinsed again and allowed to dry. Five random fields per insert were photographed with a light microscope (Leica DM 4000B) at 10x magnification and ImageJ software was used to count cells. In all experiments, data were collected from three chambers.

Cytotoxicity assays. MTS assay. U87-MG cells were seeded into 96-well plates ($1×10^3$ cells/well) and maintained overnight. Then, cells were exposed to various concentrations of Cisplatin (0-100 µM) for 72 h either under hypoxia or normoxia. Cells under hypoxia were preincubated for 24 hours in the hypoxic chamber. Hypoxia-induced resistance was measured by MTS–formazan reduction (Promega, USA) by absorbance at 492 nm and indicated as "Relative percentage of Cisplatin resistance". Three independent experiments were performed in quintuplicate.

Trypan Blue exclusion assay. Cells were plated in 60mm tissue culture plates ($5x10^4$ cells). For hypoxic conditions, cells were preincubated in the hypoxic chamber for 24 hours before treatment consisting of 20 µM Cisplatin in presence or not of 10 µM paxilline, tested under conditions of

normal and low oxygen tension for 72h. At 24h, 48h and 72h cells were trypsinized, collected, diluted 1:1 with trypan blue. Cell viability was determined by loading 10 µl of the cell suspension into countess cell counting chamber slides that were read by the countess automated cell counter (Invitrogen). Three independent experiments were performed in triplicate.

Colony formation assay. U87-MG cells (35 cells/cm^2) were plated in 60mm dishes, incubated at 37°C, 5% CO2 and let them attach overnight. Cells were treated with 1 µM cisplatin, in presence or not of 10 µM paxilline, under hypoxia or normoxia conditions. Cells under hypoxia were pre-incubated in a hypoxic chamber for 24h. Medium was replaced every 4 days. After 14 days, colonies were fixed with 4% paraformaldehyde for 15min and stained with 0.5% crystal violet (Sigma) for 2h and rinsed with water for colony visualization. Colonies were scored using an inverted microscope and images were acquired under a transilluminator. All experiments were performed three independent times in triplicate.

Immunofluorescence staining. U87-MG cells (5 x 103) were plated on chamber slides (Nunc Lab-Tek) and maintained for 5 days in DMEM supplemented with 10% FBS either under hypoxia or normoxia, in presence or not of paxilline (10 µM). For immunofluorescence staining we followed the protocol previously described by our group (Rosa et al., 2017) and incubated fixed cells overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-Sox-2 (dilution 1:200, sc-20088, Santa Cruz); rabbit polyclonal anti-Nanog (dilution 1:1000, ab21624, Abcam) and rabbit polyclonal anti-Oct4 (dilution 1:1000, ab18976, Abcam). Goat anti-rabbit Alexa-fluor 488 secondary antibody (dilution 1:1000, Life Technologies) was used and nuclei were counterstained by incubating cells with DAPI for 3 min in the dark. A Nikon Eclipse Ni motorized microscope was used to acquire the images at 10x magnification.

Immunohistochemistry. Paraffin-embedded tissues were deparaffinized, rehydrated in descending graded alcohols, incubated for 15 minutes in methanol containing 3% H_2O_2 to block endogenous peroxidase activity, and then subjected to microwave antigen retrieval for 20 minutes in sodium citrate buffer (10 mM tri-sodium citrate dihydrate, 0.05% Tween 20, pH 6.0). After preincubation in normal horse serum for 1 hour, sections were incubated overnight with mouse monoclonal anti-HIF-1α antibody (Santa Cruz, sc-13515, dilution 1:200) or rabbit polyclonal anti-BK antibody (Alomone Labs, APC-151, dilution 1:200) at 4°C in humid chamber, washed with PBS, incubated for 1 h at room temperature with biotinylated universal secondary antibody (Vectastain Universal Elite ABC kit, Vector Laboratories, Burlingame, CA), washed with PBS and then incubated with the avidin–biotin-peroxidase complex according to the manufacturer's instructions. The sections were then stained with 3-3-diaminobenzidine (ImmPACT DAB peroxidase substrate, Vector Laboratories, Burlingame, CA) as chromogen to visualize the reaction product, and were finally counterstained with hematoxylin. Images were acquired with Nikon Eclipse Ni motorized microscope system at 20X magnification.

Statistical analysis. All statistical analyses were performed using Origin v.8 software. A p-value <0.05 was considered statistically significant.

RESULTS

Hypoxia increases the activity of BK channels in U87-MG glioblastoma cells

Hypoxic cultures were obtained by 24 h incubation in a hypoxic chamber through which a mixture of gasses (95% N_2 and 5% CO_2) was flushed. Effective attainment of hypoxic conditions was probed by assessing the activation of HIF-1α in U87-MG held for 24 h under chronic hypoxia, as described above. Western blot analysis shows a marked increase of HIF-1α (Figure 1A, top). The induction of VEGF mRNA, a major target of HIF-1α, was also assessed by real time PCR analysis to corroborate the previous result (Figure 1A**,** bottom).

We next assessed if the hypoxic treatment would affect BK currents. As shown in Figure 1B, the mean BK current density in U87-MG cells grown under hypoxia was markedly higher than under normoxia. Notably, mean current fold of increase was much higher at intermediate voltages (7.8±1.2 at +80 mV, after subtraction of the leak component) as compared to more depolarized voltages (4.6±0.8 at +140 mV), indicating a leftward shift in the channel voltage dependence, hence a functional activating action of hypoxia on the BK channel. This point was further demonstrated by assessing the conductance-voltage relationship, and looking at the voltage threshold for channel activation. As evident in Figure 1D, U87-MG cell incubation under hypoxic conditions resulted in a clear shift of the voltage threshold for BK channel activation towards more hyperpolarized potentials. In order to more accurately assess this voltage shift, we fitted the experimental data with Boltzmann relationships, assuming unchanged maximal conductance and voltage steepness, and found that BK channels recorded from cells held under hypoxia displayed a 33 mV shift in the hyperpolarizing direction.

We next verified if BK current upregulation by hypoxia, in addition to a leftward shift of its activation curve, could also be due to overexpression of BK channels in U87-MG. To this end we performed expression analyses at mRNA and protein level and found that chronic hypoxia does not induce changes in the expression of the KCNMA1 gene (Figure 2A), as well as in the total BK channel protein (Figure 2B). In accordance, cytofluorimetric analysis showed no increase in the surface protein levels (indicated in Figure 2C as Mean Fluorescence Intensity, MFI). These data indicate that BK channels are not overexpressed under hypoxia in U87-MG cells, the increased BK

current likely resulting solely from a positive modulation of the channel activity by hypoxia-induced factors.

BK channels blockage inhibits hypoxia-induced migration of glioblastoma cells

Given the critical impact of chronic hypoxia on GBM cell migration (Catacuzzeno *et al.*, 2015), we verified whether BK channels were involved in the increased migratory potential of GBM cells under hypoxic conditions. Figure 3A, illustrating a wound healing assay, shows that U87-MG cells grown under hypoxia migrate faster than do cells grown under normoxia. Notably, in presence of 2 μM of the BK channel blocker paxilline the hypoxia-induced increase in the migratory ability of U87-MG cells was completely abolished (Figure 3B). Identical results were obtained in transwell migration assays, where we noticed an increased migratory potential of U87-MG cells grown in hypoxia, as compared to normoxia, and a strong inhibition of this effect upon application of paxilline (Figure 3C). We finally tested a primary GBM culture (PAL), which likewise showed a greater ability to migrate under hypoxia (about 2-fold higher than control cells in the transwell migration assay), as compared to normoxia, and a significant inhibitory effect by paxilline (Figure 3D). Notice however that, in contrast to U87-MG cells, PAL cells maintained a significant response to hypoxia in presence of paxilline. These data consistently point to BK channels as active players in the hypoxia-induced GBM cell migration.

Role of BK channels in the chemoresistance of U87-MG cells to cisplatin under hypoxia

Before testing the role of BK channels activity in the hypoxia-induced chemoresistance of GBM cells to cisplatin, we performed toxicological experiments on U87-MG cells to establish the extent of chemoresistance to cisplatin induced by hypoxia. Figure 4A left shows dose-response curves of cell viability as a function of varying concentrations of cisplatin (0-100 μM, 72 hrs treatment); cell viability, an index of cell chemoresistance, was determined with the MTS method. The relative enrichment of cells under hypoxia (blue line), as compared to cells grown under normoxia (black line), indicates that hypoxia increases chemoresistance to cisplatin. The largest difference in the percentage of hypoxia-induced resistance (about 20%) was obtained with 20 μM cisplatin applied for 72 h (Figure 4A right).

We next evaluated the role of BK channels on U87-MG hypoxia-induced chemoresistance (measured as cell viability) to cisplatin, by blocking the BK channels with paxilline. As shown in Figure 4B a hypoxia-induced chemoresistance to cisplatin could be observed on the U87-MG cell viability, that totally disappeared in presence of paxilline, a result that suggests an essential role of BK channels in the hypoxia-induced acquisition of chemoresistance.

Role of BK channels in the clonogenic potential of U87-MG cells under hypoxia

It has been shown that hypoxia also increases the ability of GBM cells to form colonies in vitro when plated at clonal densities. To address the involvement of BK channels in the hypoxia-induced

increase in the clonogenic potential of U87-MG cells, we performed a colony formation assay of U87-MG cells lasting 14 days, by culturing cells in either normoxia or hypoxia, and in presence or absence of paxilline. As found by others (Li et al., 2013a), we found that hypoxic conditions favored the clonogenic potential of U87-MG cells (Figure 5). In addition, the BK channel inhibitor paxilline did not appreciably modify the effect of hypoxia, suggesting that BK channels are not involved in the hypoxia-induced increase in the clonogenic ability of U87-MG cells. Given the role of BK channels in the hypoxia-induced chemoresistance (cf. Figure 4), we then repeated the assay for colony formation in the presence of 1 μM cisplatin. Figure 5 shows that under these conditions hypoxia favors much more the formation of U87-MG clones, and BK channel inhibition totally prevents the action of hypoxia. These data point to a fundamental role of BK channels in the capacity of U87-MG cells treated with cisplatin to form colonies under hypoxia.

Role of BK channels in the hypoxia-induced de-differentiation of U87-MG cells.

We investigated the effect of hypoxia on the expression of stemness markers in U87-MG cells. As shown in Figure 6, we observed an increased expression and the nuclear translocation of the stemness markers OCT-4 (Figure 6A), Nanog (Figure 6B) and Sox-2 (Figure 6C) in an appreciable subpopulation of U87-MG cells grown under hypoxia. This effect could be visibly prevented by the BK channel blocker paxilline. These results further confirm the important role played by BK channels in glioblastoma cells under hypoxia, that consists in favoring the acquisition of a stemness phenotype.

Expression of BK channels in hypoxic areas of human GBM tissues

Immunohistochemistry of BK channel and HIF-1α proteins was performed in order to evaluate their presence and distribution in grade IV gliomas. Normal brain cortex was included as control. In human brain cortex BK channel is currently found in the cytoplasm of neurons and neuropils, but not in astrocytes (Figure 7a). Only few cortical neurons were weakly stained for HIF-1α in the cytoplasm (Figure 7b). When we tested our glioblastoma samples with the BK antibody we could demonstrate the presence of a subpopulation of tumor cells expressing high levels of BK protein in their cytoplasm (Figure 7c and e). This subpopulation is widely diffused within the tumor but can be found clustered in angiogenic areas in some patients (see Figure 7c). Immunohistochemistry evaluation of GBM samples for HIF-1α protein showed a moderate nuclear and cytoplasmic staining in the tumor areas (Figure 7d and f). These observations emphasize the presence of BK channels in hypoxic areas of the tumor.

DISCUSSION

Our results show that, i) chronic hypoxia upregulates the BK current in GBM cells by facilitating the activation of BK channels; their expression on the plasma membrane is instead unchanged; ii) BK channel are instrumental to the increase of GBM cell migration under hypoxic conditions; iii) BK channels chemosensitize GBM cells to the action of cisplatin; iv) BK channels mediate the hypoxia-induced GBM cell de-differentiation.

Hypoxia increases BK currents in GBM cells. Following 24 h hypoxic conditioning we observed a substantial increase of voltage-evoked BK currents in U87-MG cells. The increase is not the result of increased number of channels in the plasma membrane, as we found that hypoxic U87-MG cells showed the same amount of BK channels α subunit mRNA and protein, assessed by RT-PCR and western blotting, respectively. Since BK channel subunits may reside in intracellular compartments (endoplasmic reticulum and mitochondria), we also assessed cytofluorimetrically whether chronic hypoxia would affect the trafficking of BK channel α subunits to the plasma membrane, but we found that the number of BK channels on the plasma membrane was not affected. Our electrophysiological data indicate instead that the hypoxia-induced BK current increase is caused by an increased tendency of BK channels to activate. BK current increase in hypoxic U87-MG cells is in fact markedly higher at intermediate voltages than at much higher voltages, compared to normoxic conditions, and the V1/2 significantly shifted leftward (i.e., BK channels require less voltage to be activated).

Several mechanisms could explain the increased BK channel tendency to activate following chronic hypoxia. A significant hypoxia-induced upregulation of the β1 subunit that leads to increased Ca^{2+} affinity of the α/β BK channel complex and increased channel activity was found in cerebral and pulmonary smooth muscle cells, as well as in HEK293 cells expressing both the α and β1 subunits (Hartness *et al.*, 2003; Resnik *et al.*, 2006; Ahn *et al.*, 2012; R. Zhang *et al.*, 2012; Tao *et al.*, 2015). We tested this option, but obtained negative results, with BK β1 subunit level remaining essentially unmodified under chronic hypoxia.

Increased BK channel activity in hypoxic U87-MG cells may also be caused by increased resting $[Ca^{2+}]_i$. It has indeed been shown that hypoxia is able to increase the $[Ca^{2+}]_i$ in GBM cells (Chigurupati *et al.*, 2010). This point is tenable even considering that we performed electrophysiological recordings in the whole-cell configuration, that in principle would not allow any change in the intracellular $[Ca^{2+}]_i$, as it is buffered by the dialyzing pipette solution. It has been variously shown, however, that the many cellular processes of cultured GBM cells prevent a complete dialysis of the cytoplasm in these processes even in the whole-cell configuration ((Ransom, Liu and Sontheimer, 2002); our unpublished observation). Thus BK channels' increased activity in cells grown in hypoxia may still be due to a higher mean resting $[Ca^{2+}]_i$ as compared to

cells grown in normoxia. A concomitant mechanism that could concur in the hypoxia-induced increase of BK channel activity may involve the increased levels of ROS that may be produced by the mitochondrial respiratory chain in low oxygen (Chandel *et al.*, 2000). Notably, it has been found that ROS modulate BK channel activity, although the effect appears variable, depending on the cell model used. For example, in fibroblasts the oxidative stress inducer H_2O_2 activates the BK channel activity by a mechanism involving PKC (Feng *et al.*, 2012), whereas in coronary smooth muscle cells H_2O_2-induced BK channel activation is dependent on PKG-Iα (D. X. Zhang *et al.*, 2012). In HEK293 cells overexpressing BK channels, the effect of H_2O_2 was instead activatory or inhibitory depending on the method of application: namely, a direct intracellular application, possibly involving a cysteine oxidation, led to BK channel inhibition, whereas extracellular H_2O_2 application that activates the PI3K-dependent transduction pathway led to BK channel activation (Liu *et al.*, 2010). Further experiments are needed to clarify the mechanism leading to the hypoxia-induced increase in BK channel activity.

BK channels and cell migration. It has been largely reported that BK channels have a relevant role in the migration of GBM cells, where they support cell volume changes and intracellular calcium influx. However, past literature does not appear fully consistent, with the BK channel inhibition sometimes resulting in inhibition of cell migration, while in other cases having no effect. In the effort to reconcile these apparently inconsistent results, we have recently proposed an interpretative paradigm according to which BK channels do not contribute to migration under conditions where the $[Ca^{2+}]_i$ is too low for their activation. They would instead become important in the migratory process under conditions of moderate $[Ca^{2+}]_i$ increase, insufficient as such to activate BK channels, but capable of preparing them for activation in response to $[Ca^{2+}]_i$ oscillations that typically occur in a migrating cell (Catacuzzeno *et al.*, 2015).

Along these lines, we expected that the increased activity of the BK channels resulting from chronic exposure to hypoxia would lead to a BK channel-based increase in GBM cells migration. In accordance, we observed that migration of GBM cells increased under hypoxic conditions, and this increase was prevented by preincubation with the BK channel inhibitor paxilline. Moreover, this observation was independent on the method used to assess cell migration (wound healing assay and transwell assay) and the GBM cell model. These results indicate that the BK current upregulation brought about under hypoxic conditions represents a critical step in the hypoxia-induced migration of GBM cells.

BK channels and chemoresistance to cisplatin. A recent study found that the chemoresistance of the ovarian cancer to cisplatin strongly correlates with the expression of BK channels, and that their inhibition or knockdown decreases cisplatin-induced apoptotic cell death (Samuel et al., 2016). This result, together with reports involving several ion channels in GBM chemoresistance (Su *et al.*, 2013; D'Alessandro *et al.*, 2016; Murphy *et al.*, 2016) prompted us to assess whether BK

channel activity had a role in the hypoxia-induced chemoresistance of GBM cells. Consistent with previous studies, we found that U87-MG cell viability (a measure of chemoresistance) increased significantly under hypoxia. Interestingly, this effect could not be observed following BK channel blockage. In trying to explain these data, of interest is the observation that in GBM, as in other tumors, hypoxia-induced chemoresistance is mediated by the promotion of autophagy, the process that allows regulated degradation or recycling of dysfunctional cellular constituents, in turn contrasting the apoptotic cell death observed in response to chemotherapeutic agents (Song *et al.*, 2009; Lin *et al.*, 2012; Wu *et al.*, 2015). Under the hypothesis that the BK channel activity promotes the hypoxia-induced autophagic process, paxilline treated cells under hypoxic conditions should no longer undergo authophagy, and this would lead to increased cisplatin-induced apoptotic cell death. Tests assessing the effect of BK channel inhibitors on the expression of autophagy markers are needed to verify the validity of this view.

BK channels and clonogenic potential of GBM cells. Finally, we tested the involvement of BK channels in the hypoxia-induced promotion of the clonogenic ability of GBM cells, an important property in the aggressive behavior of this tumor under hypoxic conditions. Our data indicate that BK channels have a role in the clonogenic ability of U87-MG cells neither under hypoxic nor under normoxic conditions. This result is in line with the finding that BK channels are not involved in cell cycle progression and proliferation of GBM cells (Abdullaev *et al.*, 2010). Given the role of BK channels in chemoresistance, we then evaluated the role of BK channels in the clonogenic ability in the presence of cisplatin. Under these conditions we found that hypoxia enhanced the clonogenic ability of U87-MG cells, possibly by promoting hypoxia-dependent resistance to cisplatin. In addition, cisplatin disclosed a substantial role to BK channels in the hypoxia-induced clonogenic ability, likely due to the differential role these channels exert on cell survival to the chemotherapeutic agent under normoxic and hypoxic conditions.

BK channels and GBM cells differentiation. In our recent work, we have demonstrated the involvement of BK channels in the differentiation of glioblastoma cells. In particular, BK channel block with paxilline promotes the differentiation of U87-MG cells in the form of neurospheres, during a typical differentiation assay with 10% FBS (Rosa *et al.*, 2017). The effect of hypoxia in inducing de-differentiation of glioblastoma cells was recently observed in U251 and U87-MG cells (Li *et al.*, 2013). Taking advantage of these observations, we investigated whether the pharmacological block of BK channels with paxilline would interfere with the ability of GBM cells to acquire stemness properties. Notably, in presence of the BK channel inhibitor paxilline, hypoxia was not anymore able to promote the expression of stemness markers, indicating the involvement of BK channels in the hypoxia-induced de-differentiation of U87-MG cells.

Conclusions. This study shows that chronic hypoxia functionally upregulates BK channels in GBM cells, and this upregulation substantially contributes to at least two aspects of the aggressive

phenotype that these cells acquire under hypoxic conditions, namely cell migration and chemoresistance. Based on these results, agents effective in inhibiting BK channels could well be considered as candidate coadjuvants in the pharmacological therapy against this tumor, especially if they are selective in targeting the glioma-specific isoform gBK.

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Titles and legends to figures

Figure 1. Expression of BK channels in U87-MG GBM cells under hypoxia. A) Upper panel: western blot analysis showing HIF-1α activation in U87-MG cells held in chronic hypoxia (24h). Bottom panel: RT-PCR analysis of the relative mRNA expression of VEGF in normoxia and hypoxia. **B)** Currents from a cell held in normoxia (left) and a cell held for 24 h in hypoxia (right), obtained by depolarizing voltage steps from -40 to 140 mV from a holding potential of -60 mV. **C)** Mean BK current densities vs. voltage relationships from cells held in normoxia and in hypoxia for 24h. **D)** Mean BK channel conductance vs. voltage relationships assessed from cells grown either in normoxia or hypoxia. The BK channel conductance was obtained by subtracting the leak currents from the total currents, and dividing the resulting pure BK currents for the driving force under our recording conditions. The solid lines represent the best fit of the experimental data with Boltzmann relationships having identical maximal conductance (0.5 nS/pF) and voltage steepness (21 mV), but variable half activation voltage (105 and138 mV in hypoxia and normoxia, respectively).

Figure 2. BK channels are not overexpressed in U87-MG glioblastoma cells under hypoxia. RT-PCR analysis **(A)** and western blot analysis **(B)** showing no changes in KCNMA1 mRNA levels and in total BK channel expression in U87-MG after 24 h of hypoxia, compared to normoxia. **C)** Cytofluorimetric analysis of surface BK channel protein showing that its expression (indicated as Mean Fluorescence Intensity, MFI) was not influenced by 24 h of hypoxia.

Figure 3. Effects of BK channels inhibition on the migration of U87-MG cells under hypoxia. A, B) Wound healing assay showing the percentage of closure of the wound in U87-MG cells under normoxia or hypoxia, in control conditions and in presence of paxilline. **C, D)** Transwell migration assay showing the number of migrated U87-MG cells and primary GBM cells (PAL), cultured in normoxia and hypoxia, and tested under control conditions and in presence of paxilline. Both assays highlight a markedly different effect of hypoxia in presence and absence of paxilline (*p<0.05; ** p<0.01; *** p< 0.001).

Figure 4. Effects of BK channels inhibition on the resistance of U87-MG to cisplatin. A) MTS assay showing the decrement in cell viability as function of cisplatin dosage (0-100 μM) in U87-MG cells after 72 h of hypoxia (circles) compared to normoxia (diamonds). **B)** Trypan blue exclusion assay showing the percentage of viable cells after treatment with 20 μM cisplatin in normoxia and hypoxia for 72 h, in presence or absence of 10 μM paxilline. The data show that hypoxia induces chemoresistance to cisplatin in U87-MG cells (measured as cell viability), and that BK channels blockage with paxilline prevents this effect (*** p< 0.001).

Figure 5. Effects of BK channels inhibition on the colony formation of U87-MG cells. A) Photographs showing U87-MG clones grown for 14 days under normoxia or hypoxia, in control conditions or treated with 1 μM cisplatin, in presence or absence of 10 μM paxilline. **B)** Bar plot showing the hypoxia-induced increase in the number of colonies under varying conditions (indicated). The plot highlights the increased power of U87-MG cells to form colonies under

hypoxia compared to normoxia, and the inhibition of this effect upon addition of paxilline. Data are the mean of three independent experiments (***, p< 0.001).

Figure 6. Effect of BK channel inhibition on the expression and localization of stemness markers in U87-MG cells under hypoxia. Immunofluorescence showing the positivity to OCT-4 (A), Nanog (B) and Sox-2 (C) of U87-MG cells grown for 5 days under normoxia, hypoxia and treated with 10 μM paxilline (PAX) under hypoxia. OCT-4, Nanog and Sox-2 are shown in green; DAPI in blue. Magnification 10x.

Figure 7 - BK channels expression in GBM hypoxic areas. Immunohistochemistry shows the expression of BK channels (**a,c,e**) and HIF-1α (**b,d,f**) in paraffin embedded sections of FN1 (**c,d**) and PC1 (**e,f**) GBM patients compared to normal (non tumoral) brain cortex (**a,b**).

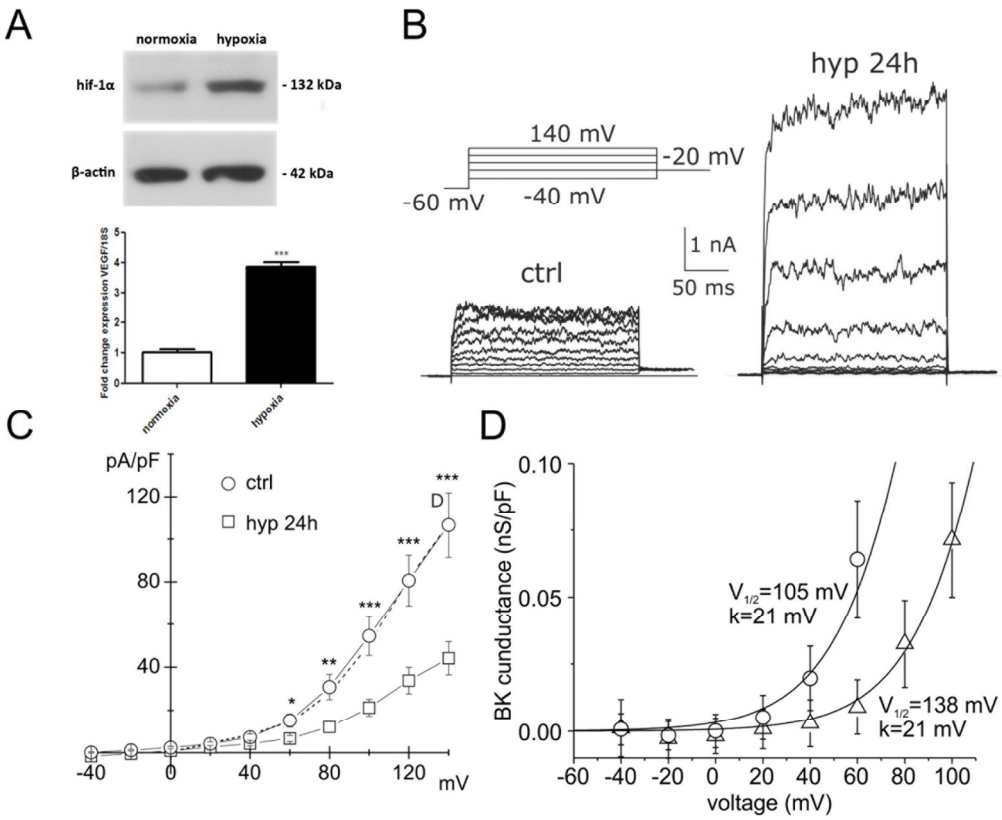

figure 1

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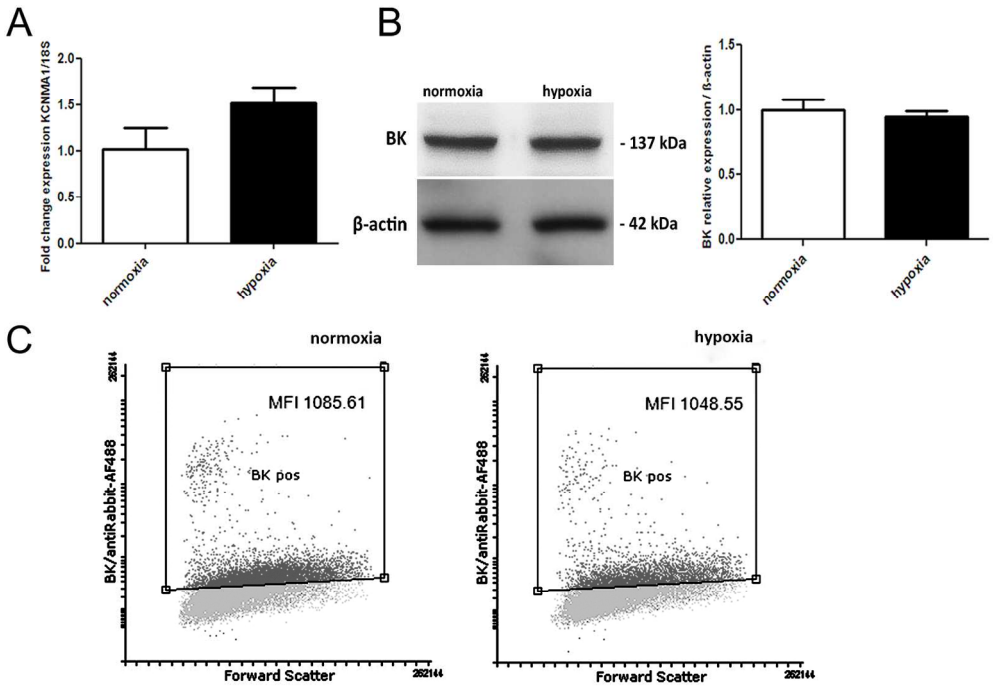

figure 2

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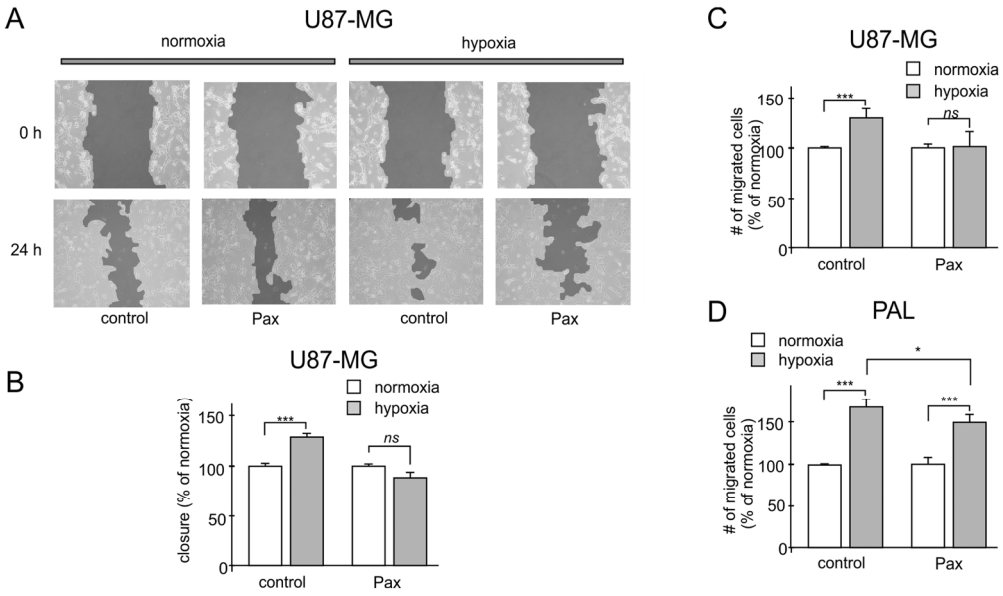

figure 3

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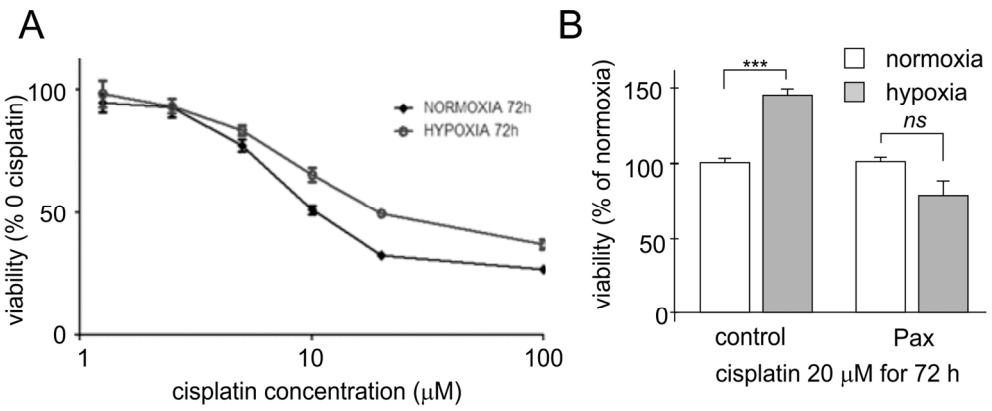

figure 4

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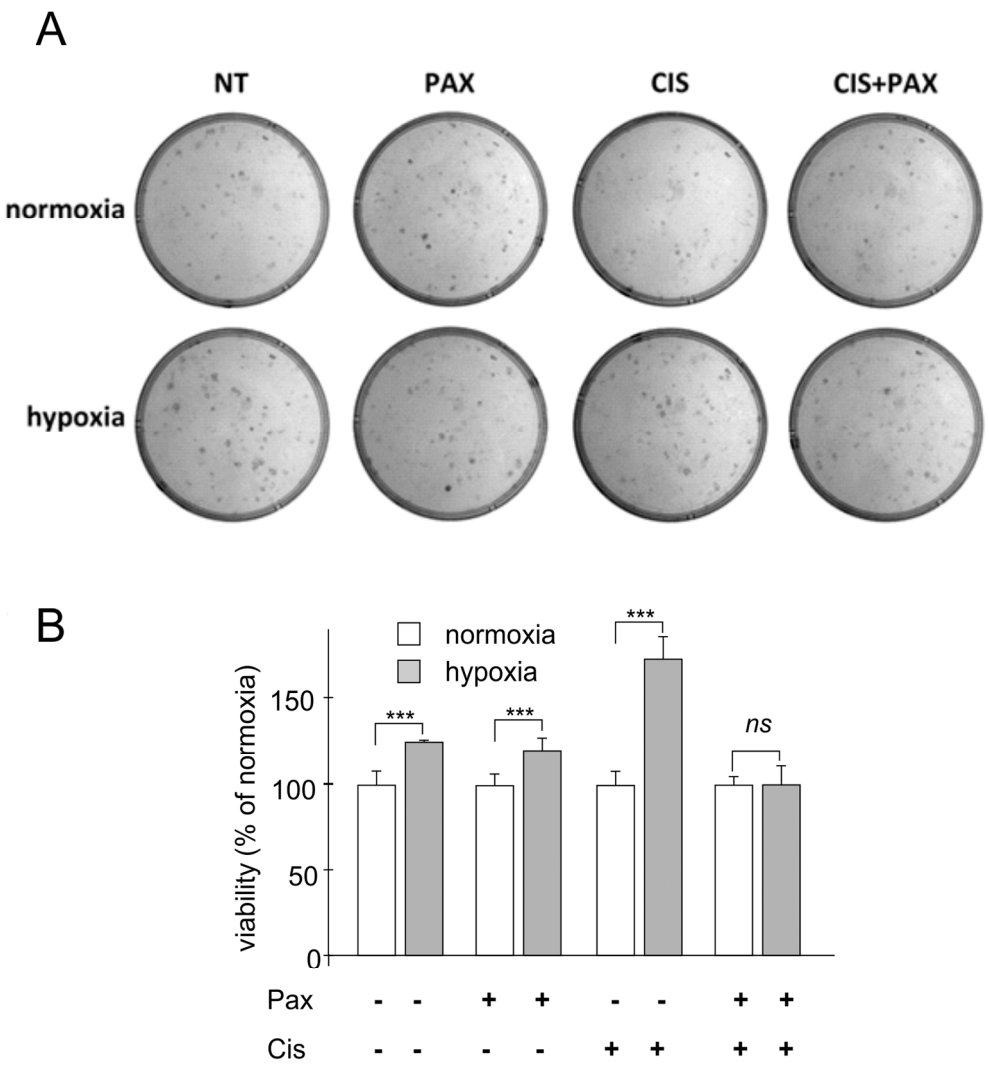

figure 5

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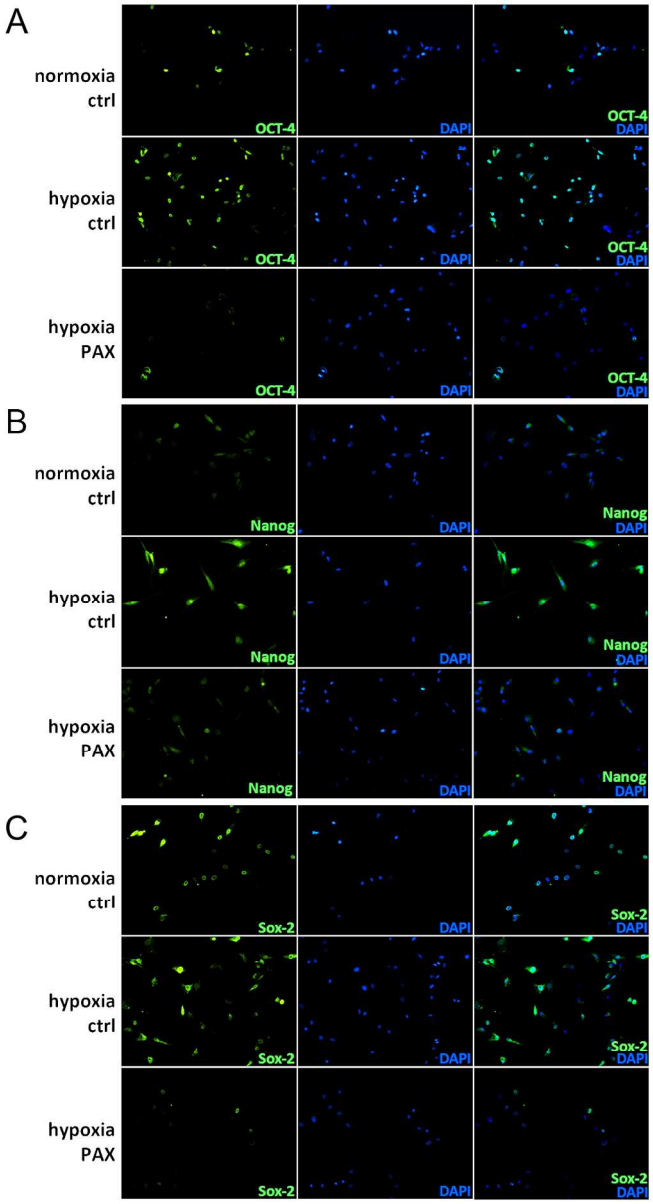

figure 6

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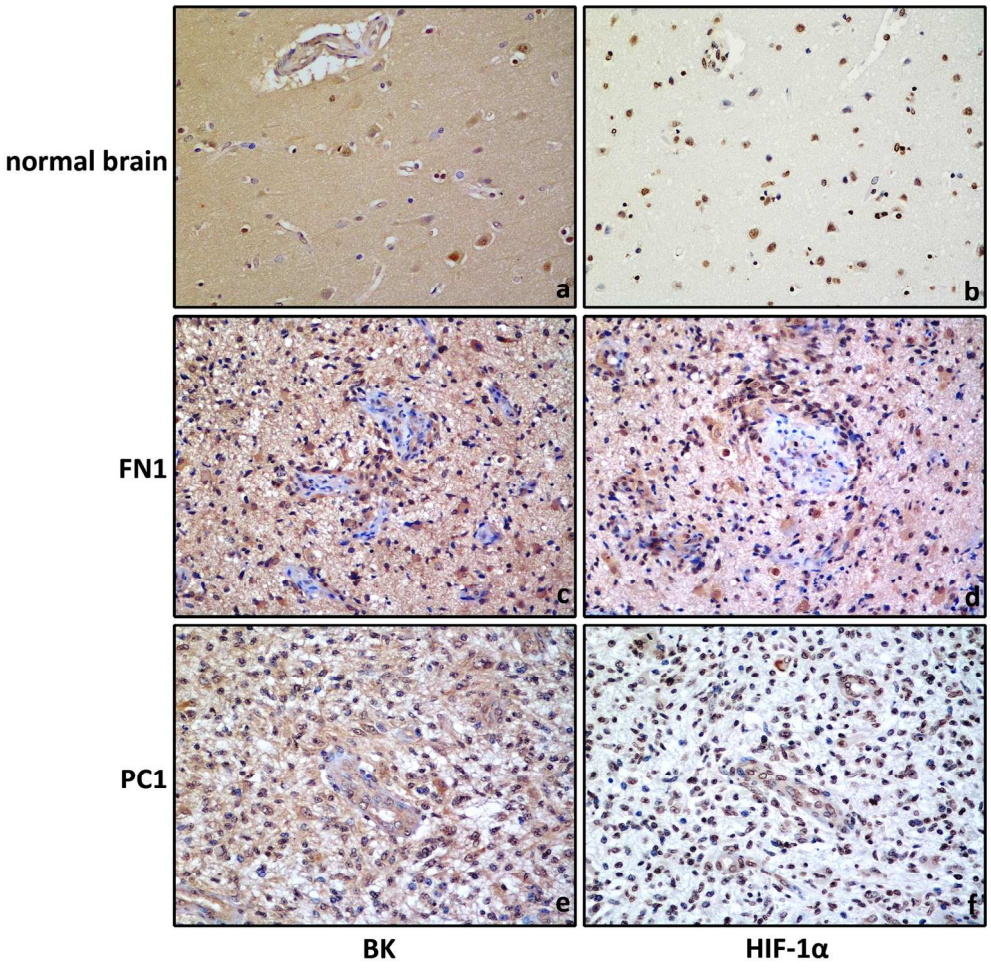

figure 7

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