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Receptor Channel TRPC6 Is a Key Mediator of Notch-Driven Glioblastoma Growth and Invasiveness

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Abstract

Glioblastoma multiforme (GBM) is the most frequent and incurable type of brain tumor of adults. Hypoxia has been shown to direct GBM toward a more aggressive and malignant state. Here we show that hypoxia increases Notch1 activation, which in turn induces the expression of transient receptor potential 6 (TRPC6) in primary samples and cell lines derived from GBM. TRPC6 is required for the development of the aggressive phenotype because knockdown of TRPC6 expression inhibits glioma growth, invasion, and angiogenesis. Functionally, TRPC6 causes a sustained elevation of intracellular calcium that is coupled to the activation of the calcineurin-nuclear factor of activated T-cell (NFAT) pathway. Pharmacologic inhibition of the calcineurin-NFAT pathway substantially reduces the development of the malignant GBM phenotypes under hypoxia. Clinically, expression of TRPC6 was elevated in GBM specimens in comparison with normal tissues. Collectively, our studies indicate that TRPC6 is a key mediator of tumor growth of GBM *in vitro* and *in vivo* and that TRPC6 may be a promising therapeutic target in the treatment of human GBM. *Cancer Res; 70(1); 418–27. ©2010 AACR.*

Introduction

Glioblastoma multiforme (GBM) is the most malignant primary brain tumor (1), Despite aggressive treatment approaches, median survival times remain less than 1 year (1). Invasion of GBM cells is the major reason for the failure of lasting success with surgical therapy and for tumor recurrence. A considerable effort has been focused on defining the mechanisms controlling GBM invasiveness and on developing therapeutic strategies aimed at reducing tumor growth and improving survival. Reduced oxygen availability (hypoxia) in the surrounding brain tissue is a major driving force behind GBM growth and aggressiveness (2). The molecular signal(s) that links tissue hypoxia to tumor aggressiveness is poorly understood.

Activation of the Notch pathway may contribute to these phenotypic changes of GBM (3). Notch plays an essential role in regulating cell fate proliferation and migration during normal development of many tissues and cell types (4). The Notch pathway consists of a family of transmembrane receptors and their ligands and Notch target transcription factors (4, 5). Binding of the ligand renders the Notch receptor susceptible to γ-secretase–mediated proteolytic cleavage, which in turn results in the release of the Notch intracellular domain (NICD) from the plasma membrane and its subsequent translocation into the nucleus. NICD interacts with the DNA binding protein CSL (CBF1, Suppressor of Hairless, Lag-1), also known as RBP-Jκ, to regulate the expression of the genes downstream of the Notch signaling, which include Hes1, Hes5, and Herp2 (4–6). In the absence of Notch signaling, CSL represses transcription of Notch target genes, and following activation by Notch, CSL is converted into a transcriptional activator and activates transcription of the same genes. The Notch signaling pathway can maintain cells in an undifferentiated state and have therefore been associated with a growing list of cancers (7). Deregulated expression of Notch receptors, ligands, and targets has been observed in many solid tumors (7–9). High-level expression of Notch1 and Jagged is associated with tumor growth and poor prognosis (7). Several members of the Notch family were found to be differentially expressed in GBMs depending on the degree of malignancy (10).

Although Notch has been associated with an oncogenic role in diverse malignancies, the Notch-regulated gene targets that are critical for the development of the aggressive phenotype remain poorly characterized. Here, we showed that inhibition of the Notch pathway in GBM blocks the hypoxia-induced upregulation of transient receptor potential 6 (TRPC6), a member cation channel of the transient receptor potential (TRPC) subfamily (11). Induction of TRPC6 promotes the aggressive phenotype by promoting a sustained

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elevation of intracellular Ca^{2+} level, which is critical for glioma proliferation and migration. Clinically, expression of Notch and TRPC6 was elevated in GBM biopsies in comparison with normal brain tissues. Collectively, these data enhance our understanding of the role of Notch and TRPC6 in human malignancies and reveal a specific molecular target that can provide the basis for developing the much needed therapies to treat malignant gliomas.

Materials and Methods

Reagents. Culture medium, serum, growth factors, antibiotics, Trizol, SuperScript II RNaseH reverse transcriptase, 4′,6-diamidino-2-phenylindole (DAPI), and Oligofectamine were obtained from Invitrogen. Other reagents include *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT) and hypoxia-inducible transcription factor-1 (Hif-1) inhibitor {3-[2-(4-adamantan-1-yl-phenoxy)-acetylamino]-4-hydroxybenzoic acid methyl ester; Calbiochem}; oleoyl-2-acetyl-*sn*-glycerol (OAG) and bromodeoxyuridine (BrdUrd; Sigma); and SK&F96365 (Tocris).

Cell culture. The human U373MG and HMEC-1 cell lines were obtained from American Type Culture Collection and Centers for Disease Control, respectively. Primary GBM was prepared by dissociation of human brain tumor patient specimens in accordance with a Florida Hospital Institutional Review Board–approved protocol.

Treatments. Hypoxic conditions were obtained by incubating cells in 100 μmol/L $CoCl_2$ (12). Cobalt has been widely used as a hypoxia mimetic in cell culture, and it is known to activate hypoxic signaling by stabilizing Hif-1α (12).

Quantitative real-time PCR. The expression levels of TRPCs, Hes1, and Hes5 were detected by quantitative real-time PCR (qRT-PCR) using the iCycler iQ (Bio-Rad) as described (13) using the primer sequences listed in Supplementary Table S1. Each sample was run in triplicate for the target gene and the internal control gene [glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)].

Gene silencing using small interfering RNA. Cells were transfected with small interfering RNA (siRNA) targeting human TRPC6 (5′-GGGCAAGGCCUUGCAGCUCdTdT-3′; siRNA-TRPC6) or human Notch1 (5′-UGGCGGGAAGUGUGUG-AAGCG-dTdT-3′; siRNA-Notch1) using Oligofectamine according to the manufacturer's instructions. The target sequences were chosen based on previous experiments testing the gene-silencing effectiveness of three siRNA duplexes (Invitrogen). A nonsilencing sequence was included as a control. Gene silencing effect was evaluated by qRT-PCR and immunoblotting as described (13).

Western blot analysis and immunofluorescence labeling. These methods are described previously (13). The following primary antibodies were used: Hif-1 (Abcam), TRPC6 (Chemicon), Notch1 intracellular domain (Val1774-NICD; Abcam), Jagged-1 (Cell Signaling), and nuclear factor of activated T cells (NFAT; Santa Cruz). Equal loading was confirmed by stripping and reprobing the membranes with β-actin (Sigma) or GAPDH (Sigma) antibodies. Densitometry quantitation was determined using the Image J software (NIH). Nuclei in immunostained specimens were visualized with DAPI (Molecular Probes).

Measurement of intracellular free Ca^{2+} concentration. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by Fluo-4 epifluorescence with excitation at 480 nm and emission at 520 nm using a PolarStar plate reader per manufacturer's instructions (Fluo-4 NW Calcium Assay Kit, Molecular Probes).

Cell proliferation assays. Cell growth was measured by using MTT cell proliferation assay (14) and by BrdUrd, which incorporates in the newly synthesized DNA and is subsequently detected by immunocytochemistry using a BrdUrd antibody (DAKO), as described previously (15).

Soft-agar colonogenic assay. Anchorage-independent growth was assessed by colony formation in soft agar as described previously (14). Colonies were counted in a blinded manner using a 10× objective on a Nikon inverted microscope. Each condition was analyzed in triplicate, and all experiments were repeated thrice. Colonies with a diameter larger than 20 μm were scored.

Matrigel invasion assay. The invasive ability of gliomas with or without treatments was examined by membrane transwell culture system as described previously (14).

Endothelial cell tube formation assay. The tube formation assay was done as previously described (14). Briefly, HMEC-1 cells were harvested and suspended in conditioned medium collected from U373 cells that were treated with $CoCl_2$ or siRNA alone or in combination. U373MG cultures left untreated were used as control.

Immunohistochemistry of human GBM specimens. Human GBM (grade 4) surgical biopsy specimens were obtained from the Preston Robert Tisch Brain Tumor Center; Duke University Medical Center and processed in accordance with the Duke University Medical Center Institutional Review Board–approved protocols. Serial sections of 11 specimens (TB# HP0308, HP0323, HP0549, HP0578, HP0591, 0430, 0444, 0445, 0456, 0457, and 0195-3691) were stained with the TRPC6 antibody as described previously (13, 16). The entire tumor was assessed by microscopy and a minimum of three separate fields were used for image collection. Images were acquired by using a Nikon Eclipse E600 fluorescence microscope and processed by using SPOT advance software (Diagnostic Instruments).

Statistical analysis. The statistical significance of differences between the means of two groups was evaluated by unpaired Student's *t* test. One-way ANOVA was used to test for differences between two or more independent groups. All statistical tests were two-sided and the level of significance was set at $P < 0.05$. Calculations were done using GraphPad Prism version 5 for Windows.

Results

Hypoxia elevates Notch signaling in glioblastomas. Clinically, hypoxia contributes to the development of aggressive phenotype and resistance to radiation and chemotherapy and is predictive of a poor outcome in numerous tumor types including GBM (2). The expression of Notch1 and it

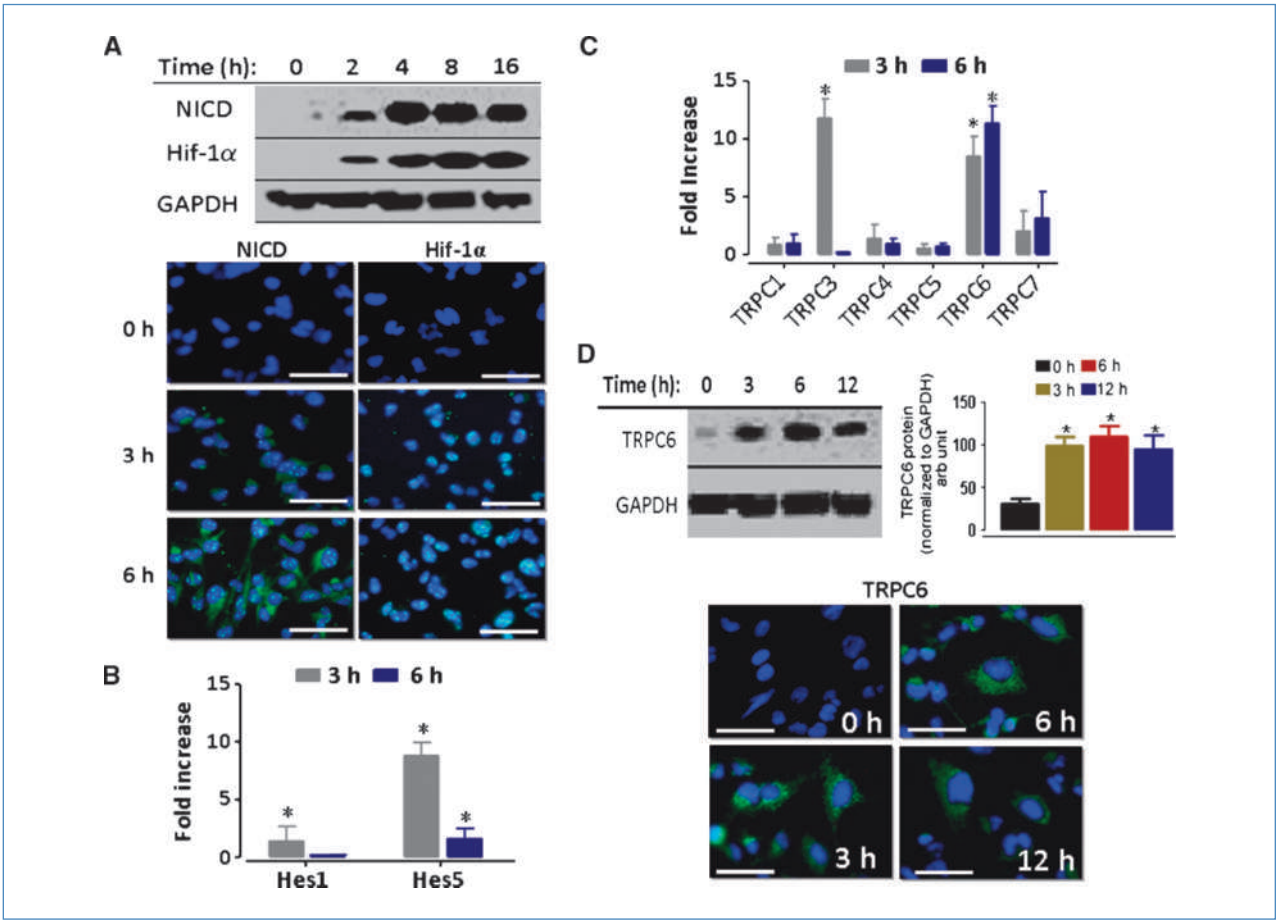

Figure 1. Effect of hypoxia on Notch signaling and TRPC expression in glioma cell line and primary human glioma. *A,* Western blotting and immunostaining showing the time course of NICD, Hif-1α, and TRPC6 protein levels in U373MG exposed to 100 μmol/L $CoCl_2$. GAPDH was used as an internal control. Immunostaining indicates the nuclear localization of Hif-1α and NICD protein. The nuclei were counterstained with DAPI. *Bar,* 15 μm. *B,* expression of Notch downstream genes Hes1 and Hes5 was measured by qRT-PCR. *Columns,* mean of three experiments, normalized to that of GAPDH used as an internal control; *bars,* SEM. *, $P < 0.01$, compared with normoxic control cultures. *C,* expression of TRPC mRNA expression was assessed by qRT-PCR. *Columns,* mean of three experiments, normalized to that of GAPDH; *bars,* SEM. *, $P < 0.01$, compared with normoxic control cultures. *D,* TRPC6 protein levels in U373MG cells exposed to 100 μmol/L $CoCl_2$ for the indicated time points were determined by immunoblotting and immunostaining. The nuclei were counterstained with DAPI. *Bar,* 10 μm. Histogram shows the densities of the TRPC6 bands. *Columns,* mean of three experiments, normalized to that of GAPDH; *bars,* SEM. *, $P < 0.01$, compared with normoxic control cultures.

ligands, Delta-like and Jagged-1, in human glioma cell lines and primary GBM cultures has previously been shown (16). Because Notch plays a role in the development and progression of various tumors, we assessed Notch activity in gliomas under hypoxia. To this end, we exposed the human glioma cell line U373MG to the hypoxia-mimicking compound $CoCl_2$ and measured by immunoblotting the level of NICD, which constitutes the activated form of Notch (4). NICD protein level was low under normoxic conditions but was rapidly upregulated by hypoxic treatment (Fig. 1*A*). Activation of the Notch pathway was also detected when U373MG cultures were subjected to hypoxia (Supplementary Fig. S1*A*). As control for the hypoxic effect, Hif-1α protein levels were increased in glioma cells (Fig. 1*A*; Supplementary Fig. S1*A*). Consistent with the elevated Notch response, the nuclear accumulation of NICD (Fig. 1*A*) and the expression of the Notch downstream gene Hes (Fig. 1*B*) were readily detected in glioma cell lines after the hypoxic switch. Specifically, Hes1 and Hes5 levels were markedly elevated in U373MG under hypoxia (Fig. 1*B*). The protein level of the Notch ligand Jagged-1 was also increased in hypoxic U373MG (Supplementary Fig. S1*B*), suggesting that ligand-dependent stimulation of the Notch receptor represents a potential mechanism by which activation of the Notch pathway is sustained in hypoxic gliomas. The Notch downstream response was also detected in primary GBM cultures following exposure to $CoCl_2$ (Supplementary Fig. S1*C*). These results indicated that Notch signaling is activated in gliomas by hypoxia.

Hypoxia induces TRPC6 expression in gliomas. Several recent studies report the involvement of TRPC channels in tumor development and malignant growth (17, 18). To explore the expression of TRPC channels in gliomas, we subjected U373MG to hypoxia and quantified TRPC transcripts by quantitative real-time PCR. TRPC6 mRNA level

was markedly increased under hypoxic compared with normoxic conditions (Fig. 1*C*; Supplementary Fig. S1*A*). TRPC3 mRNA was also transiently elevated. Expression of other TRPCs seemed less affected by hypoxia. Similar results were obtained when total mRNA was extracted from primary GBM cultures subjected to hypoxia (Supplementary Fig. S1*B* and *C*). Immunoblotting and immunofluorescence staining confirmed the induction of TRPC6 protein in U373MG following the hypoxic switch (Fig. 1*D*). No staining was observed when the primary antibody was omitted or when the antibody was blocked with the TRPC6 peptide (data not shown).

Hypoxia-induced TRPC6 expression in human malignant gliomas requires Notch signaling. To determine the involvement of Notch in hypoxia-induced TRPC6 expression in gliomas, we used the small-molecule γ-secretase inhibitor DAPT to pharmacologically inhibit the proteolytic processing of Notch to NICD (19). The level of inhibition of Notch activity was assessed by immunoblotting for NICD. Pretreatment with DAPT markedly reduced the amount of NICD (Fig. 2*A* and *B*) and substantially inhibited Hes1 mRNA expression (data not shown) in U373MG, which confirms the efficacy of DAPT in inhibiting the hypoxia-induced activation of the Notch pathway. Immunoblotting (Fig. 2*A* and *B*) and immunolabeling (Fig. 2*C*) indicated that pretreatment with DAPT greatly abrogated hypoxia-induced TRPC6 expression. No staining was observed when the primary antibody was omitted or when the antibody was blocked with the TRPC6 peptide (data not shown). To further establish that the Notch pathway is responsible for hypoxia-induced TRPC6 expression, we used siRNA to inhibit endogenous Notch1, which is known to be highly expressed in gliomas (3). Inhibition of Notch1 significantly inhibited TRPC6 protein expression in U373MG following the hypoxic switch (Fig. 2*D*),

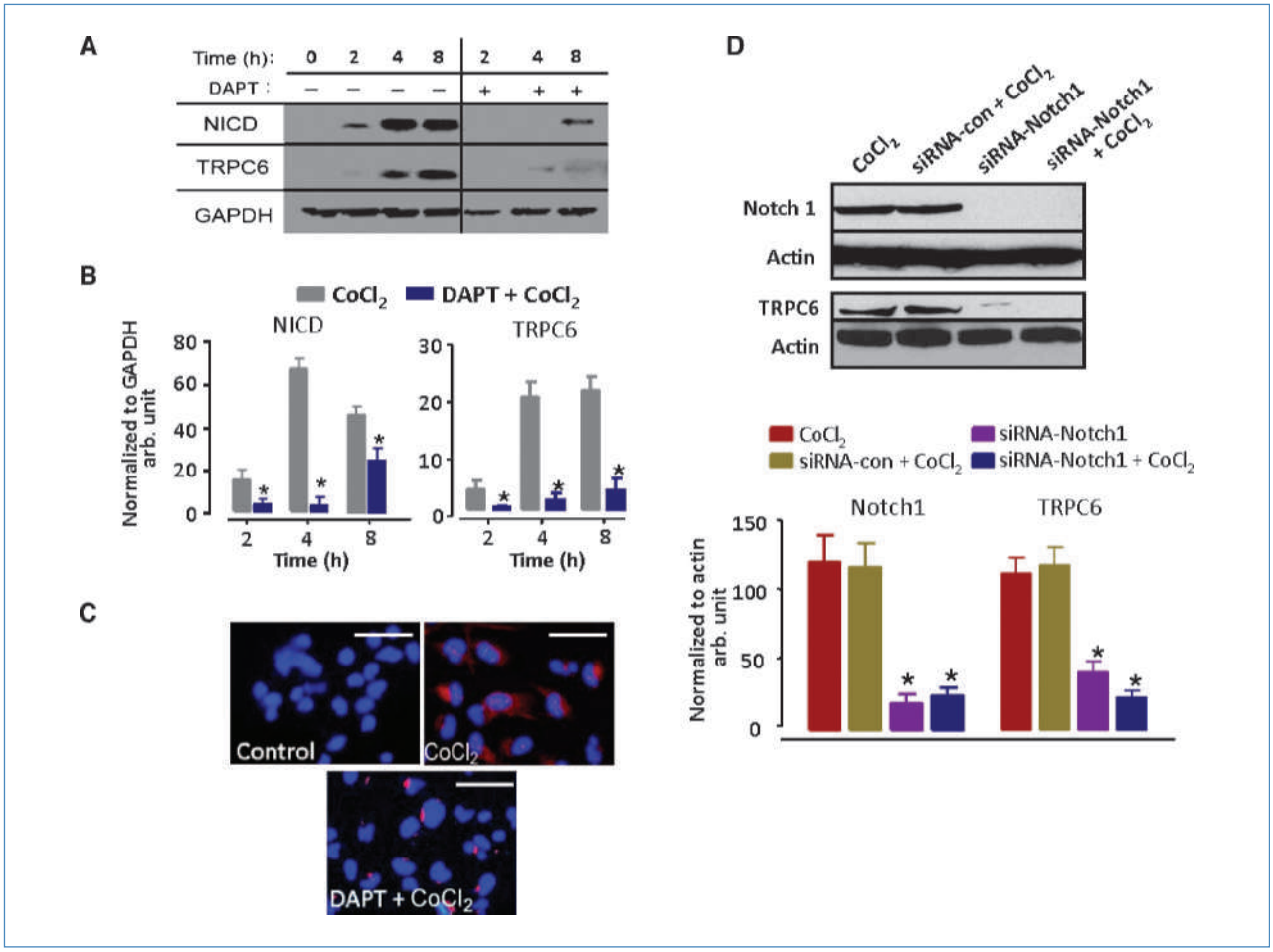

Figure 2. Hypoxia-induced TRPC6 expression in gliomas requires Notch signaling. *A,* time course of NICD and TRPC6 protein levels in U373MG cells that were pretreated with or without the γ-secretase inhibitor, DAPT (20 μmol/L), for 2 h before incubation with 100 μmol/L $CoCl_2$ for the indicated time points in the continued presence or absence of DAPT. GAPDH was used to verify equal protein loading. *B,* histograms show the density of the NICD or TRPC6 protein band normalized to GAPDH. *, $P < 0.01$, compared with normoxic control cultures. *C,* immunofluorescence labeling of TRPC6 protein in U373MG before and after treatment with 100 μmol/L $CoCl_2$ in the presence of DAPT (20 μmol/L). The nuclei were counterstained with DAPI. *Bar,* 15 μm. *D,* knockdown of Notch1 suppressed hypoxia-induced TRPC6 protein expression in U373MG cultures. Cultures were transfected with 100 pmol/L siRNA-Notch1 for 24 h and incubated with or without 100 μmol/L $CoCl_2$ for another 8 h. *Columns,* mean, normalized to that of actin; *bars,* SEM. *, $P < 0.01$, compared with cultures treated with $CoCl_2$ alone.

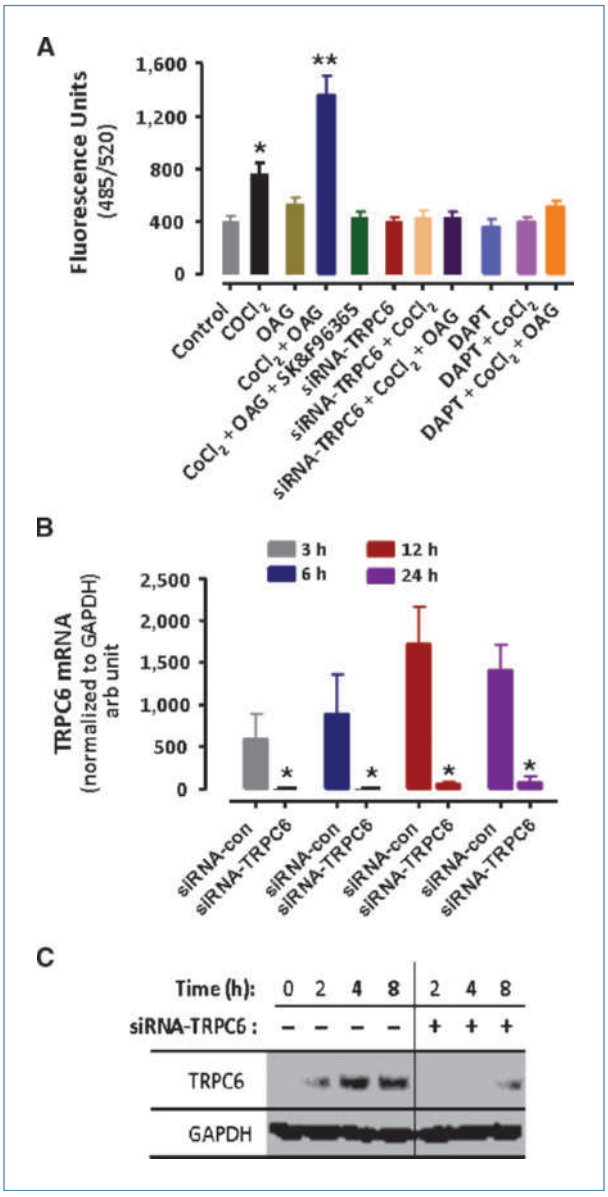

Figure 3. TRPC6 functionally augments Ca^{2+} entry in gliomas under hypoxia. *A,* treatment of U373MG cultures with 100 μmol/L $CoCl_2$ for 8 h increases basal $[Ca^{2+}]_i$ and Ca^{2+} transients evoked by 100 μmol/L OAG that were blocked by 10 μmol/L SK&F96365. Pretreatment of U373MG cultures for 2 h with 20 pmol/L siRNA-TRPC6 or 20 μmol/L DAPT blocked the hypoxia-induced increase in $[Ca^{2+}]_i$ and the OAG-induced Ca^{2+} transient. *Columns,* mean of three experiments; *bars,* SEM. *, $P < 0.05$, **, $P < 0.01$, compared with normoxic control cultures. *B* and *C,* time course of the hypoxia-induced TRPC6 transcript (*B*) and protein (*C*) levels in U373MG cultures that were transfected with 20 pmol/L siRNA-TRPC6 or siRNA-con. *Columns,* mean, normalized to that of GAPDH; *bars,* SEM. *, $P < 0.01$, compared with normoxic control cultures.

confirming that hypoxia-induced TRPC6 protein expression requires activation of the Notch1 pathway.

TRPC6 augments Ca^{2+} entry in gliomas under hypoxia. Next, we determined whether the hypoxia-induced TRPC6 expression was accompanied by a sustained increase in steady-state $[Ca^{2+}]_i$ by using Fluo-4 fluorescence spectrophotometry. Basal $[Ca^{2+}]_i$ was significantly elevated 8 hours after the hypoxia switch (Fig. 3*A*). To determine whether the expressed TRPC6 functionally augmented Ca^{2+} entry in U373MG, we applied the membrane-permeant diacylglycerol analogue OAG (100 μmol/L), which induces Ca^{2+} entry through the receptor-operated subtypes TRPC3, TRPC6, and TRPC7 (20, 21). Application of OAG evoked Ca^{2+} transients that were significantly greater in hypoxic compared with normoxic U373MG (Fig. 3*A*), suggesting that the expressed TRPC6 protein assembled into functional channels. Application of 10 μmol/L SK&F96365, a blocker of TRPCs (22, 23), blocked the OAG-evoked Ca^{2+} transients (Fig. 3*A*).

To verify that TRPC6 was mainly responsible for the increase in $[Ca^{2+}]_i$ and OAG-induced Ca^{2+} transients in hypoxic U373MG, we selectively knocked down TRPC6 expression. Transfection of U373MG with siRNA-TRPC6, but not siRNA-con, substantially blocked the hypoxia-induced expression of TRPC6 mRNA (Fig. 3*B*) and protein (Fig. 3*C*). Knockdown of TRPC6 did not alter the expression of other TRPC mRNAs (data not shown). TRPC6 knockdown inhibited the hypoxia-induced elevation of $[Ca^{2+}]_i$ and the OAG-stimulated Ca^{2+} entry (Fig. 3*A*), indicating that TRPC6, but not TRPC3 and TRPC7, is mainly responsible for the enhanced basal Ca^{2+} entry in hypoxic U373MG. Pretreatment with DAPT markedly inhibited the elevation of basal $[Ca^{2+}]_i$ and the OAG-evoked Ca^{2+} transients (Fig. 3*A*), consistent with the notion that Notch signaling mediates TRPC6-dependent Ca^{2+} entry in hypoxic U373MG. As expected, application of siRNA-TRPC6 and DAPT had no significant effect on basal (Fig. 3*A*) and OAG-evoked increase of $[Ca^{2+}]_i$ (data not shown) in U373MG maintained under normoxia.

TRPC6-mediated Ca^{2+} entry increases NFAT activation and glioma cell proliferation. Ca^{2+} signaling regulates cell growth and proliferation (24, 25). Because TRPC6-mediated Ca^{2+} entry is coupled to the activation of NFAT (26, 27), a Ca^{2+}-dependent transcription factor implicated in cell proliferation and hypertrophy-associated gene expression (26–28), we validated the role of TRPC6 in NFAT signaling in gliomas. Hypoxia increased NFAT activation, as evidenced by increased nuclear localization of NFAT (Fig. 4*A*). Because NFAT activation requires sustained elevation of $[Ca^{2+}]_i$ (28), we determined whether the TRPC6-mediated Ca^{2+} entry is important for NFAT activation in gliomas. Knockdown of TRPC6 expression significantly inhibited the accumulation of NFAT in the nucleus (Fig. 4*A*), indicating that TRPC6 is required for NFAT activation in hypoxic U373MG. Pretreatment of U373MG with FK506, an inhibitor of the Ca^{2+}-dependent calcineurin that dephosphorylates and activates NFAT (29–32), significantly attenuated the hypoxia-induced nuclear translocation of NFAT (Supplementary Fig. S2*A*), further establishing the role of TRPC6 in mediating the Ca^{2+} dependency of NFAT activation in hypoxic U373MG.

Next, we determined whether TRPC6 promotes NFAT-dependent cell proliferation following the hypoxic switch. BrdUrd incorporation was markedly inhibited in $CoCl_2$-treated cultures in the presence of siRNA-TRPC6 (Fig. 4*B*). MTT assay confirmed the antiproliferative effect of TRPC6

knockdown (Supplementary Fig. S2*B*). Similarly to TRPC6 knockdown, treatment with FK506 markedly decreased cell proliferation (Supplementary Fig. S2*B*). The decrease in cell proliferation in TRPC6 knockdown cells was not linked to cell death because no appreciable cell death was detected 72 hours after treatment with siRNA-TRPC6 (data not shown).

TRPC6 expression supports colony formation and cell invasion. Next, we determined whether TRPC6-mediated Ca^{2+} entry is involved in the malignant growth of glioma cells under hypoxia. Treatment of U373MG with siRNA-TRPC6 decreased the number of colonies (Fig. 5*A*) and the average colony size (Fig. 5*B*), indicating that hypoxia-induced TRPC6 promotes *in vitro* tumorigenesis of glioma cells.

Because Ca^{2+} signals have also been associated with cell polarization and locomotion (29, 33), we next sought to determine whether TRPC6 affects cell migration in a Matrigel-based invasion assay. Under hypoxia, treatment with DAPT substantially reduced the percentage of cells that migrated through the inserts (Fig. 5*C*), indicating that Notch inhibition impaired glioma cell migration. The anti-invasion activity associated with DAPT was mimicked by TRPC6 knockdown (Fig. 5*C*), indicating that TRPC6 modulates the migratory and invasive activity of gliomas. NFAT inhibition by FK506 markedly attenuated, but did not abolish, the Ca^{2+} dependency of glioma migration (Fig. 5*C*), suggesting that Ca^{2+} dependent activation of NFAT contributes, in part, to the hypoxia-induced increase in the metastatic potential of glioma cells.

TRPC6 expression supports angiogenesis. Hypoxia is associated with tumor growth through the formation of new blood vessels, a process called angiogenesis. Glioblastomas are among the most angiogenic of all human tumors, and the level of angiogenesis in glioblastomas is closely correlated with the degree of malignancy and patient prognosis (1). The calcineurin-NFAT pathway has been implicated in angiogenesis (30, 31), but the underlying mechanism is not clear. To determine whether the TRPC6-calcineurin-NFAT pathway plays a role in angiogenesis, we measured the

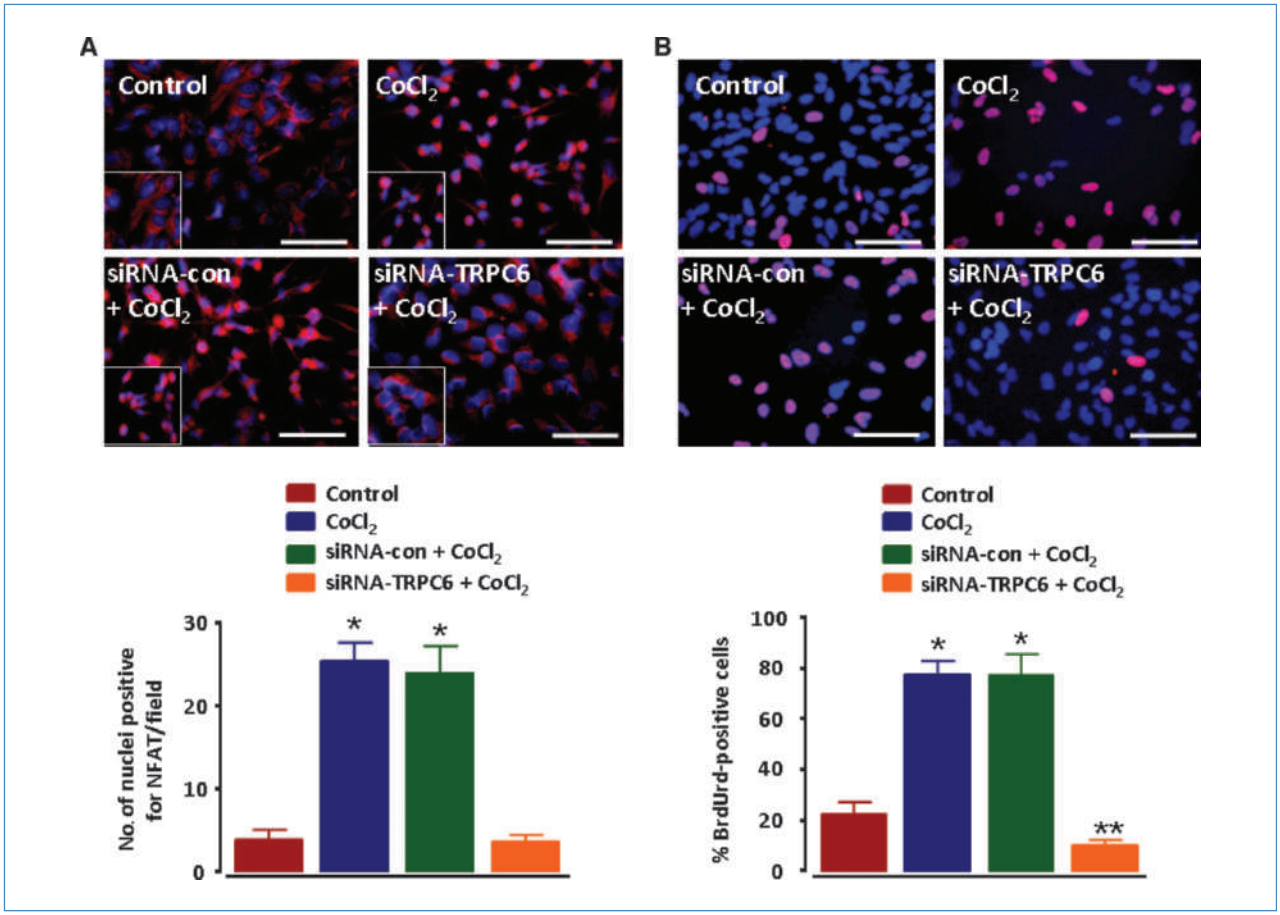

Figure 4. TRPC6 expression increases NFAT-mediated cell proliferation in gliomas under hypoxia. *A,* inhibition of hypoxia-induced TRPC6 expression blocks the nuclear translocation of NFAT. Twenty-four hours after transfection with siRNA-TRPC6 or siRNA-con, U373MG cultures were treated with $CoCl_2$ for 6 h and the nuclear translocation of NFAT was determined by immunocytochemistry. Histogram shows the number of DAPI-stained nuclei that are also labeled with NFAT. *Columns,* mean of four experiments; *bars,* SEM. *, $P < 0.01$, compared with normoxic control cultures. *B,* TRPC6 knockdown inhibits cell proliferation. Representative photomicrographs of BrdUrd labeling of U373MG cultures that were treated with $CoCl_2$ for 48 h in the presence of the indicated siRNA duplexes (20 pmol/L). Histogram shows the percentage of cells labeled with BrdUrd. *Columns,* mean ($n = 6$ wells); *bars,* SEM. *, $P < 0.01$; **, $P < 0.05$, compared with normoxic control cultures. *Bar,* 15 μm.

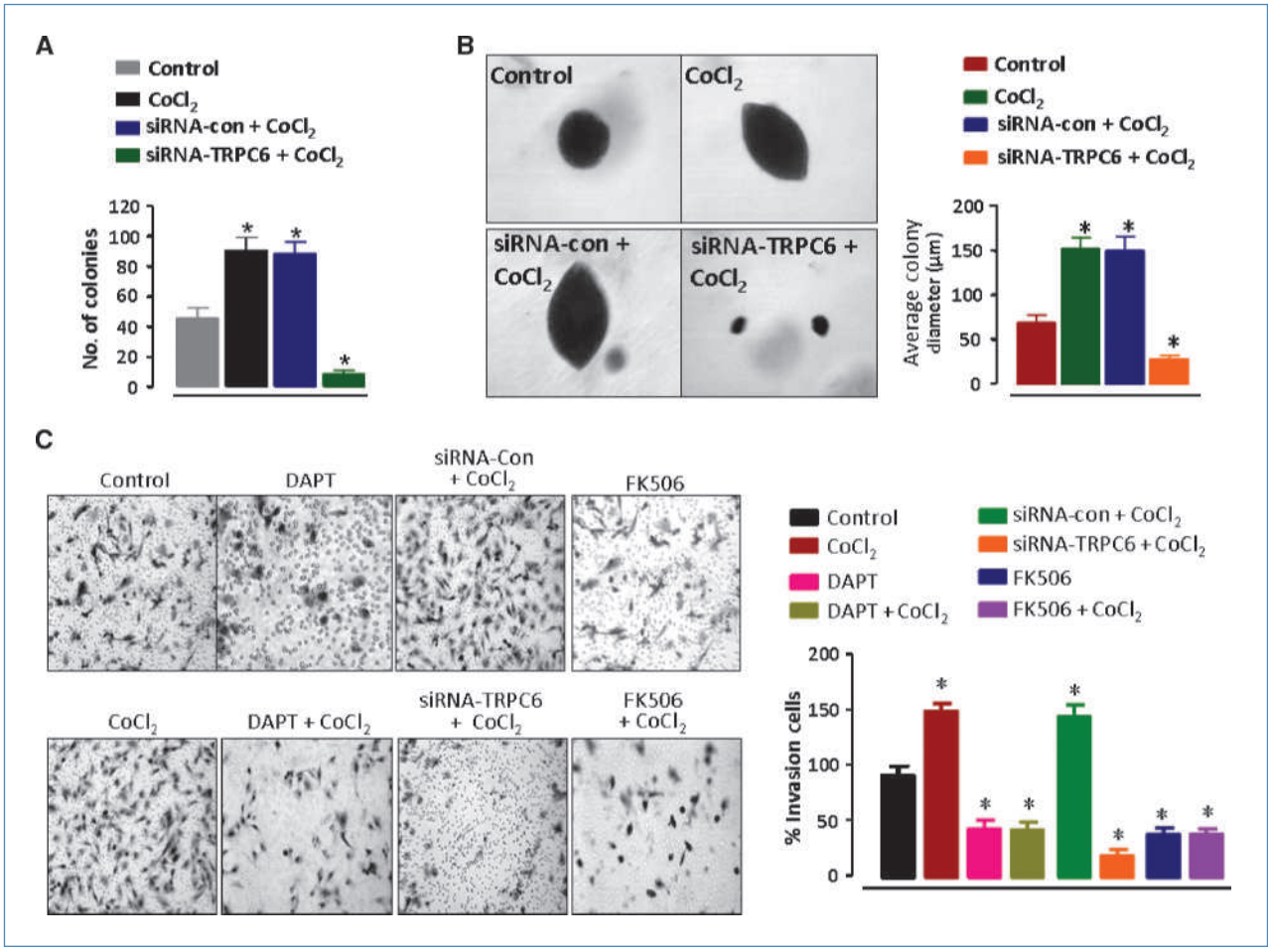

Figure 5. Role of TRPC6 in colony formation and cell invasion. *A* and *B,* suppression of hypoxia-induced TRPC6 expression or NFAT activation decreases the anchorage-independent growth of U373 MG cells. For each experiment, U373MG cultures were transfected with the indicated siRNA duplexes (20 pmol/L), plated on soft agar, and incubated for 16 d to allow colony formation. Cultures treated with siRNA-TRPC6 formed fewer (*A*) and smaller (*B*) colonies in comparison with siRNA-con. *Columns,* mean of three experiments; *bars,* SEM. *, $P < 0.01$, compared with normoxia. *C,* representative microphotographs showing the hypoxia-induced migration of U373MG in the presence of DAPT (20 μmol/L), siRNA duplexes (20 pmol/L), or FK506 (1 μmol/L) and its vehicle. The extent of cell motility is indicated by the amount of neighboring area cleared by the cells. Original magnification, ×200. Histogram shows the quantitation of the number of migrated U373MG. *Columns,* mean of five fields counted ($n = 3$ separate experiments); *bars,* SEM. *, $P < 0.05$, compared with normoxic control cultures.

effect of TRPC6 knockdown or NFAT inhibition by FK506 on the ability of hypoxic U373MG to induce endothelial cell tube formation *in vitro.* Inhibition of the hypoxia-induced TRPC6 expression and NFAT activation markedly reduced the number of branch points (Fig. 6*A*), indicating that TRPC6 is essential for the angiogenic potential of glioma cells.

Notch activation and TRPC6 expression are increased in human GBM specimens. To determine whether the Notch-induced TRPC6 expression is also seen in actual human GBMs, we performed TRPC6 immunohistochemistry on GBM specimens (grade 4) and normal brain tissues. We detected marked TRPC6 expression in GBM specimens by immunohistochemistry (Fig. 6*B*). By contrast, TRPC6 protein expression was low in the corresponding brain regions of age-matched normal subjects. No specific immunoreactivity was detected when the primary antibody was omitted or when the TRPC6 antibody was adsorbed with the TRPC6 peptide (Fig. 6*B*).

Discussion

GBM is the most common and most malignant primary brain tumor in humans. Invasion of GBM cells is the major reason for the lack of lasting success with surgical therapy and for tumor recurrence. Hence, defining the mechanism controlling invasion is essential to improving cancer survival. The low oxygen environment in the brain is positively related to GBM aggressiveness and poor prognosis (32). The role of Hif-1α in tumor growth and invasion is well established (34). Hif-1α protein was undetectable or low in U373 cells under normoxic conditions but increased markedly under hypoxia.

Similarly, Notch1 activity was low in glioma cell lines but was elevated after the hypoxic switch (Fig. 1*A*; Supplementary Fig. S1*A*). In addition to Notch1, other components of the Notch pathway were increased in glioma cells after the hypoxic switch. Specifically, the levels of Jagged-1 protein were increased under hypoxia (Supplementary Fig. S1*B*). Consistent with these findings, several members of the Notch receptor family were found to be differentially expressed in gliomas depending on the degree of malignancy (10). Although the findings presented in this study were from the U373 cell line, we observed similar results with the U118 cell line.

The Notch-regulated transcriptional targets that are responsible for the development of the aggressive and malignant phenotypes in GBM remain poorly characterized. In this study, we showed that TRPC6 is markedly upregulated under hypoxia in a manner dependent on Notch activation. Basal expression of TRPC6 is low or undetectable in U373MG (Fig. 1*D*). Pharmacologic inhibition of Notch blocked the hypoxia-induced upregulation of TRPC6 in U373MG (Fig. 2). The induction of TRPC6 expression was subtype specific because other members of TRPC subfamily were unaffected (Fig. 1*C*), suggesting that TRPC6 is the major determinant of the increase in receptor-operated Ca^{2+} entry in hypoxic U373MG. Functionally, TRPC6 increases steady-state $[Ca^{2+}]_I$, which was blocked by treatment with DAPT and siRNA-TRPC6 (Fig. 3*A*). Ca^{2+} entry in hypoxic U373MG was induced by OAG and inhibited by the TRPC blocker SK&F96365, further establishing that TRPC6 is responsible for the sustained increase in steady-state $[Ca^{2+}]_i$ in hypoxic U373MG.

Previous *in vitro* and *in vivo* studies have suggested that Ca^{2+} channels are important in growth control (35, 36). Specifically, TRPC6 channels have been implicated in cell proliferation and hypertrophic gene expression through the activation of the calcineurin-NFAT pathway in normal (26, 27) and malignant (37, 38) cells. Because glioma cells lack the expression of voltage-gated calcium channels (36) and because Ca^{2+} signaling promotes G_1-S phase transition and cell cycle progression in a variety of cell types (24, 26), the TRPC6-mediated sustained elevation of $[Ca^{2+}]_i$ and activation of the calcineurin-NFAT pathway is vital for the proliferation and malignant growth of gliomas under hypoxia (Fig. 4). Consistent with this notion, inhibition of the hypoxia-induced TRPC6 expression causes a dramatic decrease in the activation of NFAT, a transcription factor that is critical for glioma cell proliferation (39, 40).

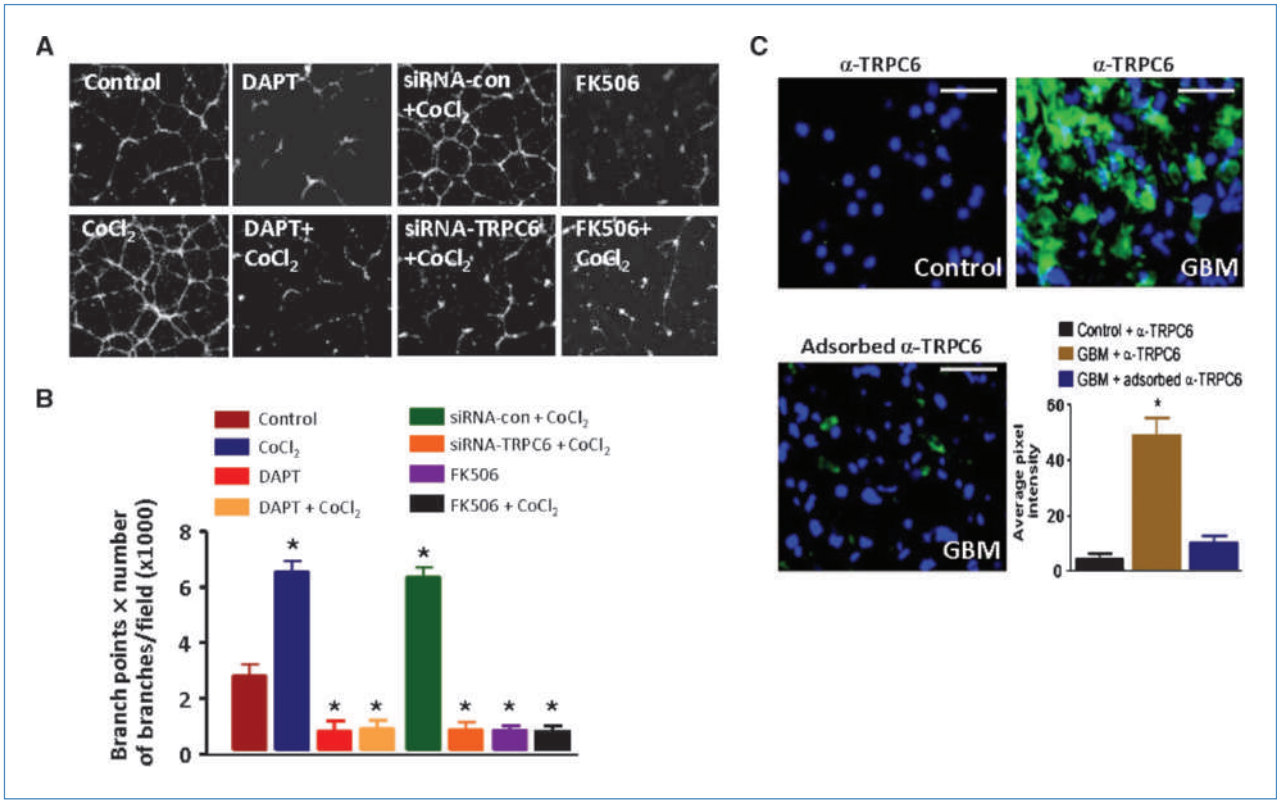

Figure 6. Role of TRPC6 in angiogenesis. *A,* representative microphotographs showing the degree of angiogenic induction in HMEC-1 cells grown in conditioned medium harvested from U373MG cultures that were treated with either the indicated siRNA duplexes (20 pmol/L) or FK506 (1 μmol/L) and its vehicle. Original magnification, ×200. *B,* the capillary length and number of branch points in HMEC-1 cultures subjected to treatments described in *A* were quantified. *Columns,* mean of quadruplicate experiments; *bars,* SEM. *, $P < 0.01$, compared with normoxic control cultures. *C,* TRPC6 staining of GBM and normal brain tissues. Sections were incubated with the TRPC6 antibody followed by Alexa Fluor 488–conjugated secondary IgG antibody and DAPI. Staining was blocked when the primary antibody was preincubated with TRPC6 peptide. Original magnification, ×400. Histogram shows the TRPC6 immunoreactivity. *Columns,* mean of 11 GBM samples; *bars,* SEM. *, $P < 0.001$. *Bar,* 15 μm.

In addition to cell growth and proliferation, Ca^{2+} signaling also plays a central regulatory role in migration (29, 33). Previous studies have shown that Notch signaling mediates hypoxia-induced tumor migration and invasion under hypoxic environment (41). Here, we showed that suppression of TRPC6 also greatly inhibited glioma cell migration and invasion in response to hypoxia (Fig. 5*C*). The molecular machinery that is responsible for cell movement is the actin cytoskeleton, which controls cell shape by assembling and disassembling itself, allowing the cell to move along the surface. Calcium-sensitive actin-binding proteins regulate the structure and dynamic behavior of the cytoskeleton. A role for TRPC6 in Rho activation and actin cytoskeleton rearrangements has been suggested (42). The TRPC6-mediated Ca^{2+} entry may contribute to invasion by promoting actin-myosin interactions and the formation and disassembly of cell-substratum adhesions that are important for glioma migration (41, 43). Recent evidence indicates that the activity of several TRPCs including TRPC6 is required for vascular endothelial growth factor–dependent angiogenesis (44) and is increased with epidermal growth factor receptor stimulation (45), suggesting that TRPCs may link growth factor response to tumor growth and invasiveness. Although Notch signaling is critical for TRPC6 upregulation, it remains to be determined whether the Notch pathway directly or indirectly, through cross talk with other transcription factors (46, 47), regulates TRPC6 transcription.

Expression of TRPC6 was higher in GBM biopsies compared with normal brain tissue (Fig. 6*B*), suggesting that TRPC6 plays a role in the malignant growth of gliomas *in vivo*. Although we examined only one type of malignant tumor in this study, our findings may also be applicable to other tumors in light of the mounting evidence for an oncogenic role of Notch in multiple types of cancers (7) including stem-like cells (48, 49).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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