

Neural Stem Cell Tropism to Glioma: Critical Role of Tumor Hypoxia

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Abstract

Hypoxia is a critical aspect of the microenvironment in glioma and generally signifies unfavorable clinical outcome. Effective targeting of hypoxic areas in gliomas remains a significant therapeutic challenge. New therapeutic platforms using neural stem cells (NSC) for tumor-targeted drug delivery show promise in treatment of cancers that are refractory to traditional therapies. However, the molecular mechanisms of NSC targeting to hypoxic tumor areas are not well understood. Therefore, we investigated the role of hypoxia in directed migration of NSCs to glioma and identified the specific signaling molecules involved. Our data showed that hypoxia caused increased migration of human HB1.F3 NSCs to U251 human glioma-conditioned medium *in vitro*. In HB1.F3 NSCs, hypoxia led to up-regulation of CXCR4, urokinase-type plasminogen activator receptor (uPAR), vascular endothelial growth factor receptor 2 (VEGFR2), and c-Met receptors. Function-inhibiting antibodies to these receptors inhibited the migration of HB1.F3 cells to glioma-conditioned medium. Small interfering RNA knockdown of hypoxia-inducible factor-1 α in glioma cells blocked the hypoxia-induced migration of NSCs, which was due to decreased expression of stromal cell-derived factor-1 (SDF-1), uPA, and VEGF in glioma cells. Our *in vivo* data provided

direct evidence that NSCs preferentially distributed to hypoxic areas inside intracranial glioma xenografts, as detected by pimonidazole hypoxia probe, as well as to the tumor edge, and that both areas displayed high SDF-1 expression. These observations indicate that hypoxia is a key factor in determining NSC tropism to glioma and that SDF-1/CXCR4, uPA/uPAR, VEGF/VEGFR2, and hepatocyte growth factor/c-Met signaling pathways mediate increased NSC-to-glioma tropism under hypoxia. These results have significant implications for development of stem cell-mediated tumor-selective gene therapies. (Mol Cancer Res 2008;6(12):1819–29)

Introduction

Malignant gliomas (e.g., glioblastoma multiforme) are the most common subtype of primary brain tumors, and glioblastoma multiforme is uniformly fatal with a mean survival of less than 1 year from diagnosis (1). Hypoxia is a critical aspect of the microenvironment in glioma and generally signifies unfavorable clinical outcome (2). Current surgical, radiation, and chemotherapy treatments do not alleviate the poor prognosis of this highly aggressive form of brain cancer, and effective targeting of hypoxic areas in gliomas remains a significant therapeutic challenge (2). Recent research indicates that neural stem cells (NSC) can specifically target invasive solid tumors, including glioma, and thus provide a novel platform for targeted delivery of therapeutic agents to tumors, resulting in significant antitumor effect (3, 4). However, it is unknown whether NSCs are able to target the hypoxic areas in glioma and which factors induced by hypoxia may be involved in NSC-glioma tropism. Here, we investigate the role of hypoxia and identify the cytokines responsible for directed migration of NSCs to gliomas.

NSC migration toward intracranial malignant tumors was first shown by Aboody et al. (5) and Benedetti et al. (6). They showed that immortalized murine and rat NSC lines could migrate great distances to sites of orthotopic human glioma xenografts in experimental animals, when injected intracranially or *i.v.*, and that such NSCs could deliver anticancer therapeutics to produce a significant tumor-killing effect (5, 6). Our recent data show that human NSCs also have the ability to target multiple tumor types and exert a therapeutic effect in melanoma brain metastases (7), disseminated neuroblastoma (8, 9), medulloblastoma (10), and glioma (11). This suggests that NSCs provide an efficient platform for targeted delivery

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and dissemination of therapeutic bioactive agents to tumors and achieve a significant antitumor response.

Trafficking of stem cells to sites of injury in ischemia models is regulated by hypoxia through hypoxia-inducible factor-1 α (HIF-1 α ; ref. 12). Similar to tissue injury, gliomas are often characterized by profound hypoxia that leads to (a) up-regulation of HIF-1 α (13) and (b) overexpression of numerous cytokines and chemoattractants, including hepatocyte growth factor (HGF), stem cell factor (SCF), stromal cell-derived factor-1 (SDF-1), urokinase-type plasminogen activator (uPA), and vascular endothelial growth factor (VEGF; refs. 14-17). Therefore, we hypothesized that cytokines induced by glioma-associated hypoxia may act as signals for attraction of NSCs. First, we compared the *in vitro* migration of HB1.F3 NSCs toward U251 human glioma cell-conditioned medium under hypoxic and normoxic conditions. We then determined the effect of hypoxia on the cytokine expression profile of U251 glioma cells and the corresponding receptors on HB1.F3 NSCs. Further, we showed that small interfering RNA (siRNA) knockdown of HIF-1 α blocks hypoxia-induced migration of NSCs. Our *in vivo* studies showed that NSCs preferentially localized to hypoxic areas in the primary tumor bed as well as the tumor border region of glioma xenografts. These data suggest that hypoxia is one of the significant factors in determining stem cell tropism to glioma.

Results

Hypoxia Induces Increased Migration of HB1.F3 NSCs

To investigate the role of hypoxia in the directed migration of HB1.F3 NSCs to glioma cells, we did *in vitro* Boyden chamber cell migration assays. Conditioned media were collected from U251 glioma cells under normoxia, hypoxia, or after treatment with desferrioxamine mesylate (DFX). DFX is an iron chelator, which increases the levels of HIF-1 α protein by promoting protein stability, without any effect on HIF-1 α mRNA level, and it can be used to mimic hypoxic conditions (18, 19).

We first investigated the migration of normoxic NSCs to conditioned medium from normoxic, hypoxic, or DFX-treated U251 glioma cells (Fig. 1). Normoxic HB1.F3 NSCs displayed highest migration to DFX-conditioned medium (23% of total cells), followed by migration to hypoxic-conditioned medium (18%), and least migration to normoxic-conditioned medium (9%). To further investigate the role of hypoxia in migration of NSCs to glioma cells, we preexposed the HB1.F3 NSCs themselves to hypoxia for 24 hours and allowed the NSCs to migrate to various conditioned media from U251 cells (normoxic, hypoxic, or DFX treated). We observed significantly increased migration of hypoxic NSCs to all conditioned medium when compared with normoxic NSCs (>100% increase of NSC migration to U251 normoxic-conditioned medium, 50% increase to U251 hypoxic-conditioned medium, and 40% increase to medium from DFX-treated U251 cells; Fig. 1). These data indicate that hypoxia leads to increased migration of NSCs to glioma cells when U251 target cells only, or NSCs only, or both cell types are exposed to hypoxia, with the latter condition showing the highest NSC migration.

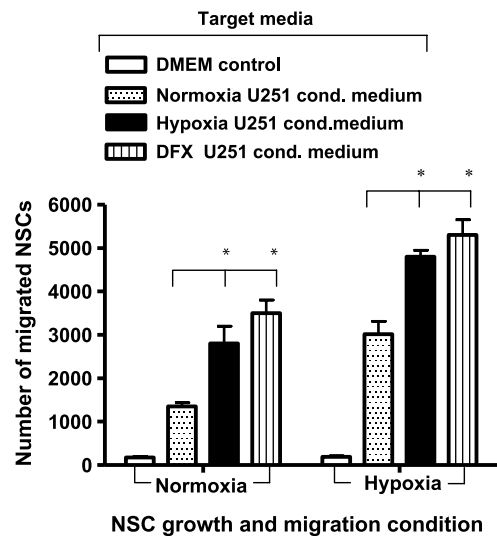


FIGURE 1. Hypoxia induces increased migration of HB1.F3 NSCs. HB1.F3 cells were preexposed to normoxia or hypoxia for 24 h. Modified Boyden chamber assays were used to determine the migration of HB1.F3 cells to different conditioned media. HB1.F3 cells (1.5×10^4) were added to the top chambers. The NSC migration assays were done under normoxia or hypoxia, matched to the normoxic or hypoxic preexposure conditions of NSCs. The number of migrated NSCs was quantified by CyQuant fluorescence cytometry. Columns, average of cell migration in triplicate wells; bars, SD. *, $P < 0.05$, compared with normoxic cultures. Similar data were obtained in three independent experiments.

Effect of Hypoxia on Cytokine Expression in Glioma Cells

To identify factors that are secreted by tumor cells and contribute to NSC-tumor tropism, we used quantitative real-time PCR to screen normoxic, hypoxic, or DFX-treated U251 cells (0- to 30-hour culture) for mRNA expression of various cytokines. As shown in Fig. 2A, we detected up-regulation of SDF-1 and uPA mRNA in U251 cells following exposure to hypoxia or treatment with DFX when compared with U251 cells exposed to normoxia (18- and 24-hour culture), with peak expression levels at 24 hours after treatment. The HGF mRNA level increased ~20% at 18 hours after hypoxia or DFX treatment. The expression of SCF mRNA under hypoxia and DFX treatment increased ~50% at 6 hours, ~20% at 12 hours, followed by a decrease at 18 hours, when compared with normoxia. However, expression of VEGF mRNA showed a continued up-regulation with increasing time of hypoxia or DFX treatment (~10-fold or ~12-fold increase at 30 hours) when compared with normoxia. Thus, both hypoxia and DFX treatment resulted in up-regulation of SCF, SDF-1, uPA, and VEGF mRNA in a time-dependent manner (Fig. 2A). Because most cytokines that we investigated showed a significant increase at 24 hours of hypoxia or DFX treatment, we chose 24-hour treatment of U251 cells to collect conditioned medium for our cytokine antibody array and NSC migration experiments. The antibody array data showed that levels of secreted SDF-1 and VEGF were up-regulated in U251-conditioned medium collected after hypoxia or DFX treatment (Table 1; Fig. 2B and C). No significant differences were observed in the expression of HGF and SCF as a result of normoxia, hypoxia, or DFX treatment of U251 cells. uPA protein level, as measured by ELISA, increased ~60% after hypoxia or DFX treatment when compared with normoxia condition (Fig. 2D).

Effect of Hypoxia on Expression of Cytokine Receptors in HB1.F3 NSCs

Because cytokines mediate the migration of NSCs to glioma cells by binding to their corresponding receptors on NSCs, we

investigated whether hypoxia can induce changes in the expression of cytokine receptors in HB1.F3 NSCs. We determined the levels of the following receptors: c-Met (receptor for HGF), c-Kit (SCF), CXCR4 (SDF-1), uPAR

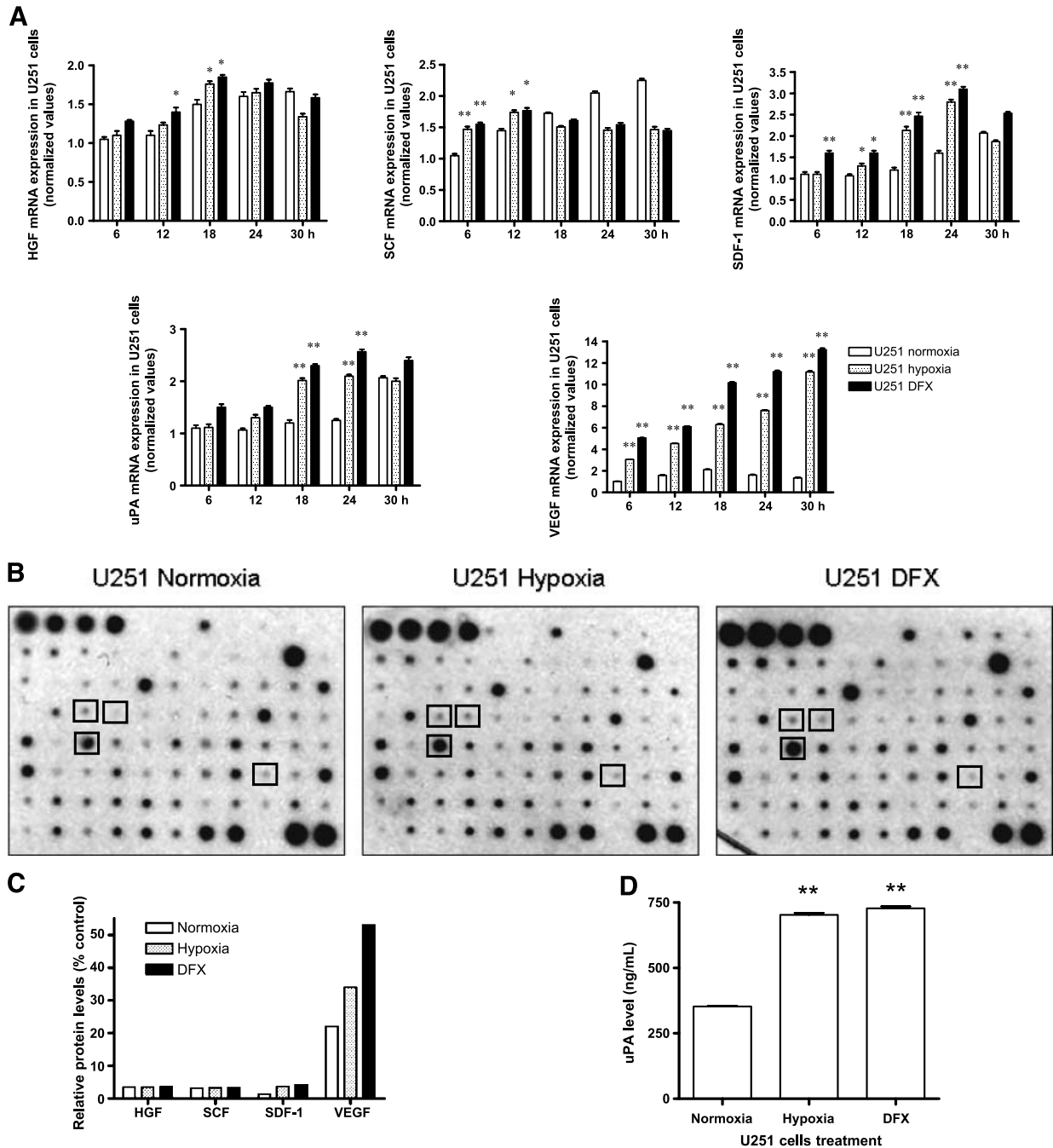


FIGURE 2. Hypoxia up-regulates the expression of cytokine mRNAs and proteins in U251 cells. U251 cells were serum starved and exposed to normoxia, hypoxia, or DFX treatment for 6, 12, 18, 24, or 30 h followed by extraction of total RNA. Real-time reverse transcription-PCR (RT-PCR) was used to assess mRNA expression of HGF, SCF, SDF-1, uPA, and VEGF. **B.** Cytokine protein profiles of U251-conditioned medium collected after exposure to normoxia, hypoxia, or DFX for 24 h as detected by Cytokine Antibody Array. **C.** Plotted data display the relative protein levels of HGF, SCF, SDF-1, and VEGF in U251-conditioned medium from normoxia, hypoxia, or DFX-treated cultures. SDF-1 and VEGF protein levels were increased after hypoxia or DFX treatment when compared with normoxia-conditioned medium. **D.** Increased level of uPA protein in U251-conditioned medium after hypoxia or DFX treatment measured by ELISA assay is shown compared with normoxia condition. Columns, mean value of triplicate wells; bars, SD. Similar data were obtained in three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Table 1. Map of the Cytokine-Specific Antibodies Spotted onto RayBio Cytokine Antibody Array V Protein Chip (RayBiotech)

	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO- α
2	I-309	IL-1 α	IL-1 β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12 p40p70	IL-13	IL-15	IFN- γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1 β
4	MIP-1 δ	RANTES	SCF	SDF-1	TARC	TGF- β 1	TNF- α	TNF- β	EGF	IGF-1	Angiogenin
5	Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ck β B-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3 α	NAP-2	NT-3
8	NT-4	Osteoprotegerin	PARC	PIGF	TGF- β 2	TGF- β 3	TIMP-1	TIMP-2	Neg	Pos	Pos

(uPA), and VEGFR2 (VEGF). We found significantly greater mRNA expression for all receptors investigated, except for c-Kit, after exposure of NSCs to hypoxia or DFX when compared with normoxic condition (Fig. 3A). At the protein level, flow cytometry data showed increased expression of c-Met, CXCR4, uPAR, and VEGFR2, but no change in c-Kit, after exposure of NSCs to hypoxia (Fig. 3B).

Hypoxia-Induced Migration of HB1.F3 Stem Cells Can Be Blocked by Neutralizing Antibodies

To determine whether HGF/c-Met, SDF-1/CXCR4, uPA/uPAR, and VEGF/VEGFR2 signaling molecules were necessary for NSC tropism to glioma, we used function-blocking antibodies against SDF-1 ligand and antibodies against receptors c-Met, CXCR4, uPAR, and VEGFR2 in cell migration assays. Anti-SDF-1 antibody inhibited the migration of NSCs to U251 normoxia- and hypoxia-conditioned medium in a dose-dependent manner (10-40% and 20-60% for normoxia and hypoxia, respectively; Fig. 4A). The neutralizing antibodies to receptors c-Met, CXCR4, uPAR, and VEGFR2 inhibited the migration of NSCs by 30% to 60% and 60% to 80% in normoxic and hypoxic U251-conditioned medium, respectively (Fig. 4B). These data further support the role of the above signaling molecules in determining the tropism of NSCs to glioma.

Silencing of HIF-1 α in Glioma Cells Blocks the Hypoxia-Induced Migration of NSCs

We did siRNA-mediated knockdown of the transcription factor HIF-1 α to examine whether HIF-1 α was involved in the increased migration of NSCs to glioma cells under hypoxic conditions. Transfection of U251 cells with HIF-1 α -specific siRNA led to decreased levels of HIF-1 α mRNA (80% reduction) when compared with control (scrambled siRNA or untransfected U251 cells; Fig. 5A). We did Western blotting to determine the HIF-1 α protein levels of normoxic, hypoxic, or DFX-treated U251 cells after siRNA transfection. HIF-1 α siRNA transfection resulted in reduced amounts of HIF-1 α protein in U251 cells under all conditions (normoxia, hypoxia, or DFX; Fig. 5B).

We then investigated the effect of HIF-1 α siRNA on the expression of cytokines in U251 cells under normoxic and hypoxic conditions. HIF-1 α siRNA inhibited the hypoxia-induced expression of SDF-1, uPA, and VEGF mRNAs, but it did not affect the cytokine levels in normoxic U251 cells

(Fig. 5C). This suggests that hypoxia-induced expression of these cytokines is under the control of HIF-1 α , whereas the cytokines present in normoxic cells are expressed independently of HIF-1 α .

To further investigate the function of HIF-1 α in NSC migration to glioma, we did NSC migration assays with conditioned medium from normoxic and hypoxic U251 cells transfected with HIF-1 α siRNA. We found that siRNA-induced silencing of HIF-1 α expression in U251 cells resulted in significantly decreased hypoxia-induced NSC migration (Fig. 5D). As expected based on the cytokine levels (Fig. 5C), HIF-1 α siRNA did not affect the migration of NSCs to conditioned medium from normoxic U251 cells (Fig. 5D). Taken together, these data suggest that HIF-1 α is a key factor in hypoxia-induced NSC migration to glioma cells by affecting the expression of multiple cytokines in U251 cells.

NSC Targeting of Glioma In vivo

To investigate the role of hypoxia in NSC tropism to glioma *in vivo*, U251 glioma cells were implanted into the brain of nude mice. Two months after implantation of tumor cells, chloromethylbenzamido-1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI)-labeled HB1.F3 NSCs were injected stereotactically into the hemisphere contralateral to the tumor. Four days after injection of NSCs, pimonidazole was administered i.p. 20 minutes before euthanasia of the mice. Pimonidazole has proven to be an effective marker for hypoxia in multiple tumor types (20). Immunohistochemical analysis revealed that the U251 glioma implants contained hypoxic regions that were defined by pimonidazole staining (Fig. 6B). Within the tumor, we observed considerable numbers of NSCs associated with the hypoxic areas, with fewer NSCs detected in the normoxic areas of the tumor (Fig. 6B). The tumor edge and the adjacent brain parenchyma also contained large numbers of CM-DiI-labeled NSCs (Fig. 6C). Further, the tumor edge and the hypoxic areas within the tumor were associated with high levels of SDF-1 (Fig. 6C). SDF-1 may be produced by reactive astrocytes that are present around gliomas (21, 22). Because HB1.F3 NSCs were injected into the contralateral normal brain parenchyma, NSCs can be expected to enter into the tumor bed through the tumor-brain interface. Indeed, we detected the largest numbers of NSCs at the tumor edge as well as in the hypoxic tumor areas in which many cytokines are up-regulated, including SDF-1. Collectively, these findings suggest that the tumor edge and the hypoxic regions in glioma are preferentially targeted by NSCs.

Discussion

We have previously shown the inherent tumor-tropic property of NSCs and their use as cellular vehicles for effective delivery of therapeutic agents to invasive tumors, including glioma, melanoma brain metastases, medulloblastoma, and disseminated neuroblastoma. Their abilities to track infiltrating tumor cells and localize to distant tumor microfoci make NSCs attractive gene therapy vehicles with promising clinical potential (3, 4). A major obstacle for current glioma therapies is ineffective delivery of drugs, especially to hypoxic tumor areas, which are not well vascularized, resulting in limited drug penetration (23). Here, we investigated whether HB1.F3 NSCs can target such “hard-to-reach” hypoxic areas in glioma, and if

so, which cytokines are responsible for the NSC targeting. Identification of hypoxia-induced cytokines involved in NSC tropism will be important for the development of optimal NSC-based glioma therapies.

A limited number of stem cell attractants and cytokines emanating from solid tumors, including glioma, have been identified (24-27). However, the cytokines produced by hypoxic areas in glioma, which are responsible for NSC attraction, had not been defined. It was also unknown which receptors on the surface of NSCs mediate the targeting of hypoxic regions of glioma. Here, we provide direct evidence, for the first time, that NSCs display increased migration to hypoxic glioma cells *in vitro* and that NSCs preferentially target the hypoxic glioma

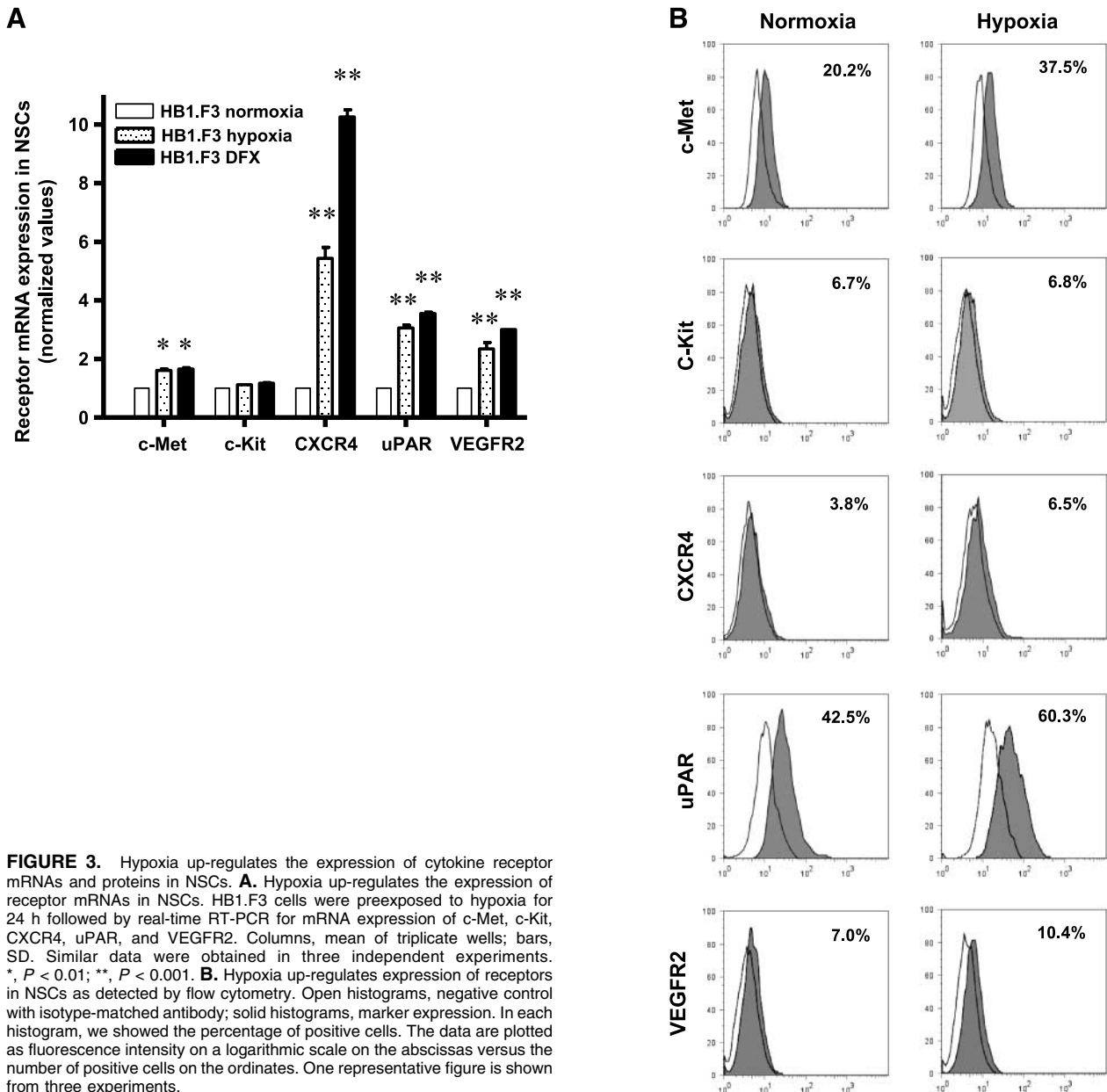


FIGURE 3. Hypoxia up-regulates the expression of cytokine receptor mRNAs and proteins in NSCs. **A.** Hypoxia up-regulates the expression of receptor mRNAs in NSCs. HB1.F3 cells were preexposed to hypoxia for 24 h followed by real-time RT-PCR for mRNA expression of c-Met, c-Kit, CXCR4, uPAR, and VEGFR2. Columns, mean of triplicate wells; bars, SD. Similar data were obtained in three independent experiments. *, $P < 0.01$; **, $P < 0.001$. **B.** Hypoxia up-regulates expression of receptors in NSCs as detected by flow cytometry. Open histograms, negative control with isotype-matched antibody; solid histograms, marker expression. In each histogram, we showed the percentage of positive cells. The data are plotted as fluorescence intensity on a logarithmic scale on the abscissas versus the number of positive cells on the ordinates. One representative figure is shown from three experiments.

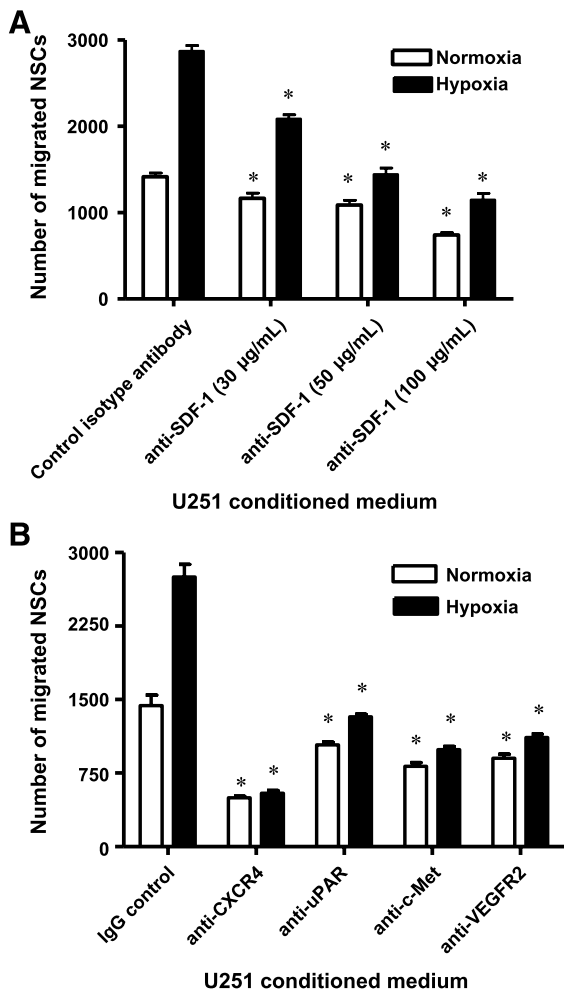


FIGURE 4. Hypoxia-induced migration of HB1.F3 NSCs is blocked by function-inhibiting antibodies. **A.** Hypoxia-induced migration of HB1.F3 NSCs is blocked by SDF-1 function-inhibiting antibody. Conditioned media from normoxic or hypoxic U251 cells were preincubated with SDF-1 function-inhibiting antibody for 2 h at room temperature, and then HB1.F3 NSCs were allowed to migrate to these conditioned media. **B.** Hypoxia-induced migration of HB1.F3 NSCs is blocked by cytokine receptor function-inhibiting antibodies. HB1.F3 NSCs were preincubated with receptor function-inhibiting antibodies or isotype-matched control antibodies for 30 min at room temperature, and then the cells were allowed to migrate to conditioned medium from normoxic or hypoxic U251 cells. The number of migrated NSCs was quantified by CyQuant fluorescence cytometry. Columns, average number of migrated cells in triplicate wells; bars, SD. *, $P < 0.01$, compared with normoxic-conditioned medium. Similar data were obtained in three independent experiments.

regions *in vivo*. Of the cytokines investigated, we found that hypoxia resulted in up-regulation of SDF-1, uPA, and VEGF mRNA and protein in U251 cells. Furthermore, the corresponding receptors of these cytokines were increased on HB1.F3 NSCs under hypoxia. Importantly, by using function-inhibiting antibodies, we showed a significant reduction of hypoxia-induced NSC migration to glioma. Figure 7 depicts a schematic model of NSC targeting to glioma and the cytokines involved. As mentioned above, SDF-1, uPA, and VEGF, as well as HGF, are secreted by glioma. The highest levels of these

cytokines are likely produced in the hypoxic tumor regions. In addition to glioma cells, reactive astrocytes and other stromal cells located in and around the tumor may also release multiple cytokines. The important role of SDF-1/CXCR4 in the migration of NSCs has been shown in models of ischemic brain injury, where SDF-1 is up-regulated in reactive astrocytes (28). Further, hypoxia-activated rat astrocytes display increased attraction of neural progenitor cells *in vitro*, and this effect is mediated by VEGF, SCF, SDF-1, and MCP-1 (29). These findings, together with our data, suggest that a combined effect of cytokines released by hypoxic tumor and by reactive astrocytes at the tumor edge mediates attraction of NSCs. The HGF/c-Met, SDF-1/CXCR4, uPA/uPAR, and VEGF/VEGFR2 ligand/receptor pairs have been implicated in glioma progression, invasion, and angiogenesis (14, 17, 30). Understanding the role of these cytokines and receptors in NSC-to-glioma targeting will help in designing exogenous NSCs with overexpression of optimal cytokine receptor combination, allowing highly effective NSC migration and drug delivery to hypoxic tumors. Our cytokine array data suggest that multiple cytokines other than the ones investigated here are also expressed in glioma cell-conditioned medium (e.g., RANTES, IFN- γ , TGF- α , and TGF- β ; Table 1; Fig. 2B). Further work will be needed to determine the role of these cytokines in NSC-to-glioma tropism.

HIF-1 is a transcription factor that plays a crucial role in cellular responses to hypoxia in normal and pathologic tissues, including tumors (31, 32). Hypoxic stimulation leads to the accumulation of HIF-1 α protein, which interacts with HIF-1 β and translocates to the nucleus, resulting in transcription of downstream target genes. More than 100 putative target genes for HIF-1 have been identified, including many genes involved in cell survival, migration, invasion, and angiogenesis (31, 33, 34). Among gliomas, there is a strong correlation between HIF-1 α expression, tumor grade, and tumor vascularization (31, 35). HIF-1 α expression is highest in the hypoxic areas immediately adjacent to necrotic foci in a subpopulation of glioma cells known as pseudopalisading cells (36). Our data show that siRNA-mediated knockdown of HIF-1 α in glioma cells led to significant inhibition of hypoxia-induced NSC tropism, which was due to decreased expression of multiple cytokines in glioma cells. This suggests that HIF-1 α is a key factor in hypoxia-induced NSC targeting to glioma. Because HIF-1 is a “master regulator” of many genes involved in tumors, it will be an important target for NSC-based glioma therapies.

The hypoxic environment in solid tumors renders tumor cells more resistant to radiation and chemotherapy, which correlates with tumor recurrence and poor patient prognosis (31, 37). Hypoxia has been identified as a major factor in radiation resistance of glioma because the low oxygen tension in hypoxic cells prevents the radiation-induced DNA damage from becoming permanent (38, 39). Hypoxic cells are also resistant to most chemotherapeutic drugs (37). The reasons for such resistance include the distance of hypoxic cells from blood vessels, poor diffusion of drugs, and up-regulation of multidrug resistance genes (23, 40). Given the central role of hypoxia in resistance of tumors to chemotherapy and radiation, targeting of hypoxic tumor cells is an attractive therapeutic strategy (31, 41). There are several clinical trials aimed at overcoming treatment failure because of tumor hypoxia (41, 42). We expect

that use of NSCs for direct delivery of anticancer drugs to hypoxic glioma will allow maximal concentrations of therapeutics at these sites, resulting in eradication of resistant hypoxic tumor cells.

Materials and Methods

HB1.F3 NSC Line

The HB1.F3 NSC line was derived from telencephalon of a human fetus of 15 wk of gestation and was immortalized using a retrovirus encoding the *v-myc* gene (43, 44). The primary cells were obtained in accordance with the Guidelines of the Anatomical Pathology Department of Vancouver General Hospital, with permission to use fetal tissue granted by the Clinical Research Screening Committee Involving Human Subjects of the University of British Columbia. The HB1.F3 NSC line is a well-characterized, clonal cell line, which is nontumorigenic and multipotent in that cells from this line can

be induced to differentiate into neurons, oligodendrocytes, and astrocytes (24, 45, 46). HB1.F3 NSCs were grown in DMEM with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 6% CO₂.

Hypoxia Treatment

HB1.F3 and U251 cells were cultured to a density of ~80% confluence in 10% FBS DMEM and then transferred to starvation medium (2% FBS for HB1.F3 cells and 0% FBS for U251 cells). The cells were then exposed to normoxia (ambient oxygen tension), hypoxia (1% O₂), or DFX (150 μmol/L) treatment for 24 h in a HEPA incubator (Thermo Forma). DFX was used as a hypoxia-mimicking agent.

Conditioned Medium

U251 human glioma cells (American Type Culture Collection) were maintained as adherent cultures in DMEM supplemented

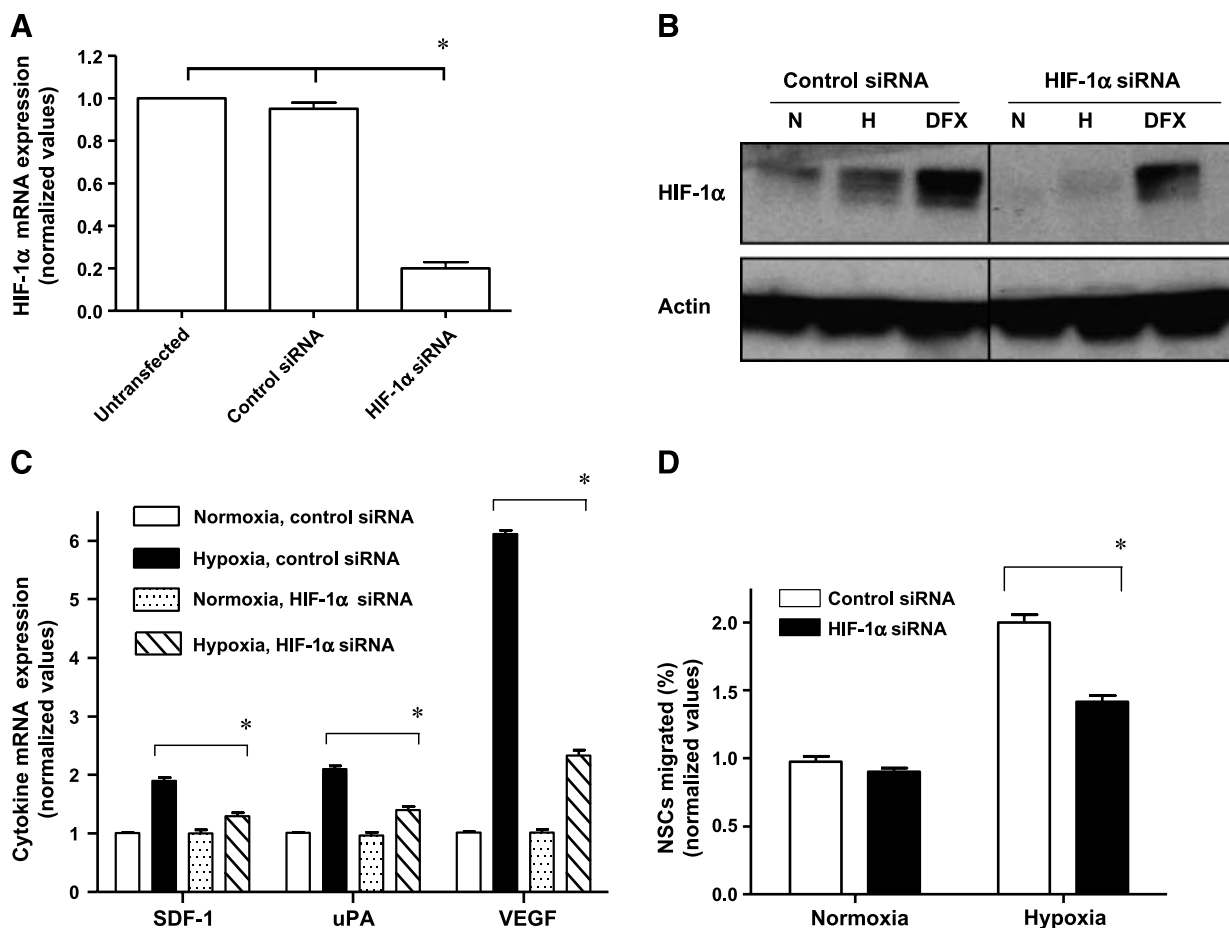


FIGURE 5. Silencing of HIF-1α in glioma cells blocks the hypoxia-induced migration of HB1.F3 NSCs. **A.** HIF-1α siRNA resulted in knockdown of HIF-1α mRNA expression in U251 cells as detected by quantitative real-time RT-PCR. **B.** HIF-1α siRNA blocked HIF-1α protein expression in U251 cells. U251 glioma cells were transfected with HIF-1α siRNA and treated with normoxia (N), hypoxia (H), or DFX. Western blotting was done to detect HIF-1α protein expression. Full-length blots are presented in Supplementary Fig. S1. **C.** HIF-1α siRNA blocks the hypoxia-induced cytokine mRNA expression. U251 glioma cells were transfected with HIF-1α siRNA and treated with normoxia, hypoxia, or DFX. Total RNAs from these cells were analyzed for cytokine mRNA expression by quantitative real-time RT-PCR. Columns, normalized fold cytokine mRNA expression (triplicates) in HIF-1α siRNA-treated cells; bars, SD. *, $P < 0.01$, compared with control siRNA-treated cells. **D.** HIF-1α siRNA blocks the hypoxia-induced migration of HB1.F3 cells. U251 cells were transfected with control or HIF-1α siRNA followed by additional incubation under normoxia or hypoxia for 24 h and collection of conditioned medium. Migration of HB1.F3 cells to conditioned medium was tested in Boyden chamber assays. Columns, average of cell migration in triplicate wells; bars, SD. *, $P < 0.05$, compared with conditioned medium derived from control siRNA-treated U251 cells.

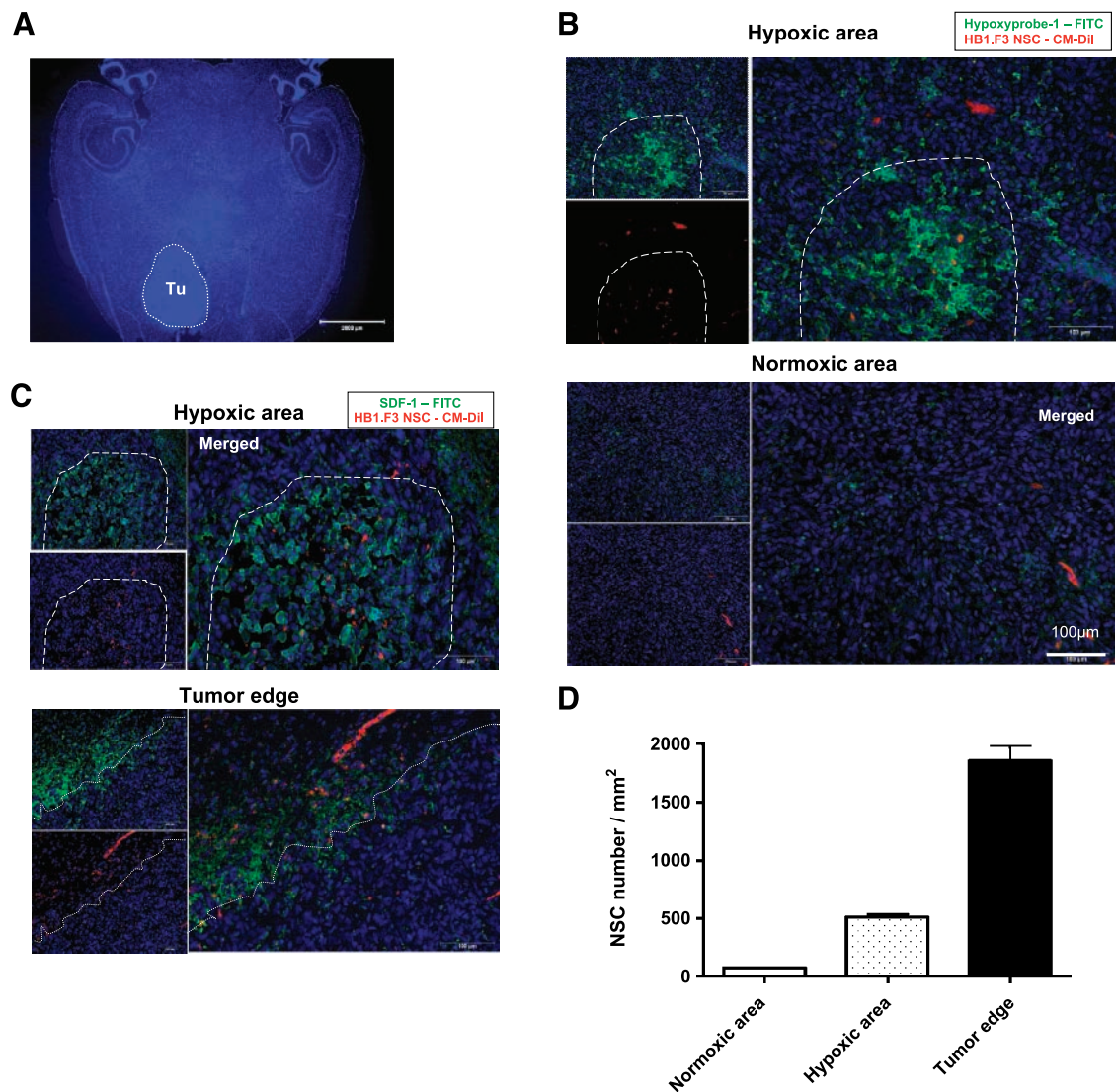


FIGURE 6. NSC targeting of glioma *in vivo*. **A.** 4',6-Diamidino-2-phenylindole staining for whole brain. Dotted line, location of glioma (*Tu*). **B.** Distribution of CM-Dil-labeled HB1.F3 NSCs (*red*) in the hypoxic (outlined by *broken lines*) and normoxic areas of glioma xenograft, as detected by Hypoxyprobe-1 (*green*). Right, merged image of CM-Dil-labeled NSCs and Hypoxyprobe-1 staining. Scale bar, 100 μ m. **C.** Localization of CM-Dil-labeled HB1.F3 NSCs (*red*) to SDF-1-rich regions (*green*) in hypoxic tumor areas (*dashed lines*) and the tumor edge (*dotted lines*). Right, merged image of CM-Dil-labeled NSCs and SDF-1 staining. Scale bar, 100 μ m. **D.** Average number of migrated NSCs in the normoxic and hypoxic areas and at tumor edge as quantified by fluorescent imaging of CM-Dil signal (*red*). $P < 0.01$, compared with normoxic areas.

with 10% FBS at 37°C in humidified atmosphere containing 6% CO₂. To obtain conditioned medium, U251 cells were grown on 100-mm plates to ~80% confluence. Cultures were rinsed thrice with serum-free medium and incubated for another 24 h in serum-free DMEM. Conditioned media were collected, cleared by centrifugation (10 min, 600 \times *g*), and stored at -80°C.

In vitro Boyden Chamber Cell Migration Assay

HB1.F3 NSCs were maintained in normoxic conditions or preexposed to hypoxia for 24 h. All migration assays were done using 96-well Boyden chambers (Neuroprobe). Lower wells were filled with either U251-conditioned medium (serum-free) or DMEM as a control. HB1.F3 cells (1.5×10^4) were added to the top chambers. After 4 h at 37°C under normoxic or hypoxic

conditions (matched to the NSC preexposure conditions), the cells that migrated to the lower membrane surface were detached by accutase solution (Innovative Cell Technologies) and transferred to a V-bottomed 96-well plate. After centrifugation, migrated cells were stained using CyQuant GR fluorescent dye (Chemicon) and counted with a fluorescence microplate reader (Molecular Devices). All experimental conditions were replicated a minimum of three times, and all experiments were done at least three times.

siRNA Knockdown of HIF-1 α

HIF-1 α siRNA was purchased from Santa Cruz Biotechnology. The HPRT-S1 siRNA, which served as positive control, and scrambled siRNA, which served as negative control, were

purchased from IDT, Inc. U251 glioma cells were transfected with HIF-1 α siRNA using X-tremeGENE siRNA transfection reagent (Roche). The medium containing the transfection complexes was replaced with fresh medium and the cells were incubated under normoxic or hypoxic conditions, starting at 24 h after transfection. The cell migration assays, real-time PCR, and Western blotting were done 48 h after siRNA transfection.

Quantitative Real-time Reverse Transcription-PCR

Total RNA was extracted after cells were grown under normoxic conditions or preexposed to hypoxia or DFX. The RNeasy Mini kit (Qiagen) was used to isolate total RNA from HB1.F3 and U251 glioma cells according to the manufacturer's instructions. Random primers, ThermoScript reverse transcriptase (Invitrogen), and 1 μ g of total RNA were used for cDNA synthesis. Relative quantification of mRNA expression was done with the SYBR Green PCR Master Mix using an ABI PRISM 7700 thermal cycler (Applied Biosystems). The primer sequences for the genes of interest are listed in Table 2. Each sample was normalized to actin as an endogenous reference. Relative quantification of target gene expression was calculated by the comparative threshold cycle (C_t) method.

Measurement of Cytokine Protein Level

Cytokine Antibody Array V protein chip was used for measurement of cytokine protein levels in U251 glioma-conditioned medium according to the manufacturer's instructions (RayBiotech). Cytokine array membranes were blocked by incubation with the blocking buffer at room temperature for 30 min and incubated with 1 mL of U251-conditioned medium at 4°C overnight. Membranes were washed thrice with Wash

Buffer I and twice with Wash Buffer II at room temperature for 5 min per wash and incubated with biotin-conjugated antibodies at room temperature for 2 h. Finally, the membranes were washed, incubated with horseradish peroxidase-conjugated streptavidin at room temperature for 2 h and with detection buffer for 2 min, and exposed to X-ray film for 30 s (Kodak, Inc.). The exposed films were digitized and the relative cytokine levels were compared after densitometry analysis using NIH ImageJ 1.36b software. Total intensity of signal spots was calculated by multiplying the area with mean signal intensity of the spot. The relative protein levels were obtained by subtracting the background signal and normalizing to the total mean intensity of the positive controls on the same membrane. Level of uPA protein in U251-conditioned medium was measured using IMUBIND uPA ELISA kit according to the manufacturer's protocol (American Diagnostica, Inc.).

Flow Cytometry Assay

Cells from each cell line were resuspended in ice-cold staining buffer (PBS supplemented with 2% FBS) and incubated with Fc-block solution for 5 min at 4°C. Subsequently, cells were stained with fluorescein phycoerythrin-conjugated antibodies against CXCR4, c-Kit, c-Met, uPAR, VEGFR2, or the appropriate isotype control (R&D Systems) at 4°C for 30 min, after which cells were washed twice with staining buffer. Samples were analyzed on a Guava EasyCyte flow cytometer (Guava Biotechnologies), and the data were processed with FlowJo7 software.

In vivo Tumor Tropism Studies

Tropism of the human HB1.F3 NSCs to orthotopic human glioblastoma xenografts was shown by implanting tumor cells (U251) into the brains of 8-wk-old female athymic nude mice. Animals were anesthetized (132 mg/kg ketamine and 8.8 mg/kg xylazine) and received stereotactically guided injections of 2×10^5 tumor cells in 2 μ L of PBS through a 30-gauge Hamilton syringe into the right forebrain (5, 7). Two months after tumor cell injection, HB1.F3 NSCs labeled with CM-Dil (Molecular Probes) were stereotactically injected into the hemisphere contralateral to the tumor using the same coordinates. After an additional 4 d, the hypoxic marker Hypoxyprobe-1 (pimonidazole; Chemicon) was injected i.p. (60 mg/kg, 20 min before euthanasia), and then mice were perfused intracardially with 4% paraformaldehyde in PBS. The brains were harvested and the Hypoxyprobe-1 kit was used for detection of hypoxia in tumors. The brains were also processed for immunohistochemical detection of various cellular/tissue antigens.

Immunohistochemistry

For immunohistochemical staining of tissues, standard protocols were done using primary antibodies against SDF-1 (R&D Systems), diluted 1:50 (10 μ g/mL final concentration) in blocking buffer (5% bovine serum albumin, 3% normal horse serum, 0.1% Triton X-100). Secondary staining was done using biotinylated anti-mouse antibodies (2 μ g/mL in blocking buffer; Vector) followed by detection with avidin-FITC (1 μ g/mL). Adjacent tissue sections were stained with anti-pimonidazole antibody from the Hypoxyprobe-1 kit (1:200 dilution in PBS)

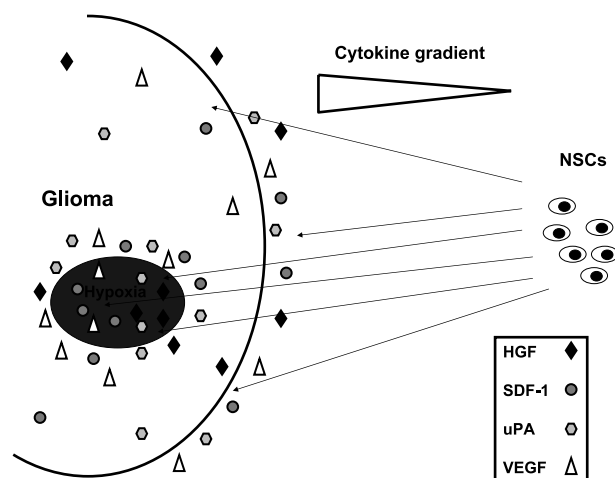


FIGURE 7. Schematic model of NSC-to-glioma targeting. Numerous cytokines, including HGF, SDF-1, uPA, and VEGF, are secreted by glioma. High levels of these cytokines are likely produced in the hypoxic tumor regions. In addition to glioma cells, reactive astrocytes and other stromal cells located in and around the tumor may also release cytokines. The cytokine gradients are sensed by NSCs, which then migrate to the glioma. Large numbers of NSCs are observed in the hypoxic tumor regions as well as at the tumor edge. Note: the relative amounts of various cytokines indicated in the schematic figure may not reflect the actual *in vivo* cytokine levels.

Table 2. Primers for Quantitative Real-time RT-PCR

	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product length (bp)
HGF	CATCAAATGTCAGCCCTGGAGTT	CCTGTAGGTCTTTACCCCGATAGC	44
SCF-1	GGCAAATCTCCAAAAGACTACA	GCCTTCAGAAATATTTGAAAACCTTG	152
SDF-1	GTGTCACCTGGCGACACGTAG	TCCCATCCCACAGAGAGAAG	263
uPA	TTGCTCACCACAACGACATT	GGCAGGCAGATGGTCTGTAT	94
VEGF	CCAGCACATAGGAGAGATGAGCTT	TCTTTCTTTGGTCTGCATTACAT	63
c-Met	TGATGATGAGGTGGACACA	ATTTTGGCAAGAGCAAAGA	149
c-Kit	CGCTGGGATTTTCTCTGC	TCACAGATGGTTGAGAAGAGCCT	72
CXCR4	CAAGGCCCTCAAGACCACA	CCCAATGTAGTAAGGCAGCCAA	70
uPAR	GCCCAATCCTGGAGCTTGA	TCCCTTGCAGCTGTAACACT	63
VEGFR-2	CGGCTCTTTCGCTTACTGTCT	AGCATGGAAGAGGATCTGGACT	143

overnight at 4°C followed by staining with a FITC-conjugated secondary antibody (Chemicon). Fluorescence microscopy was done using a Nikon TE2000-U fluorescence microscope at 488 nm (FITC) and 568 nm (CM-DiI). Images were captured and processed using SPOT 4.0 (Diagnostic Instruments, Inc.). The numbers of CM-DiI-labeled NSCs in hypoxic and normoxic areas and at the tumor edge were counted in three mouse brains (five sections per brain). The sizes of the relevant areas (square millimeter) were determined using NIH ImageJ software.

Western Blot Analysis

Total U251 cell lysate was separated in an 8% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 1 h at room temperature and then incubated overnight at 4°C with anti-HIF-1 α antibody (Santa Cruz Biotechnology) diluted 1:200 in 5% nonfat milk in Tween 20-TBS. Blots were then stained with horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology) followed by enhanced chemiluminescence for signal detection (Perkin-Elmer Life Sciences).

Statistics

All data were analyzed with GraphPad Prism 4.0 software. Data were presented as mean \pm SD. Statistical differences between two groups were evaluated using two-way ANOVA. *P* values of <0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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