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ABSTRACT

The invasive nature of glioblastoma (GBM) is one important reason for treatment failure. GBM stem/progenitor cells retain the migratory ability of normal neural stem/progenitor cells and infiltrate the brain parenchyma. Here, we identify Rai (ShcC/N-Shc), a member of the family of Shc-like adaptor proteins, as a new regulator of migration of normal and cancer stem/progenitor cells. Rai is expressed in neurogenic areas of the brain and its knockdown impairs progenitor migration to the olfactory bulb. Its expression is retained in GBM stem/progenitor cells where it exerts the same promigratory activity. Rai silencing in cancer stem/progenitor cells isolated from different patients causes significant decrease in cell migration and invasion, both in vitro and in vivo, providing survival benefit. Rai depletion is associated with alteration of multiple-signaling pathways, yet it always leads to reduced expression of proinvasive genes. STEM CELLS *2012;30:817–832*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive among brain tumors. The inability to treat these tumors is mainly due to the infiltrative nature of GBM cells. Invasive tumor cells disseminate within normal brain parenchyma, escaping surgical resection and resisting to localized radiation and chemotherapy exposure, thus allowing tumor recurrence [1]. GBMs are maintained by a small fraction of tumor cells, defined GBM initiating cells or cancer stem cells, which share common characteristics with neural stem cells, such as extensive self-renewal, migration, and differentiation abilities [2–4]. Since glioma invasion is clinically relevant, the identification of both specific markers and underlying molecular mechanisms supporting cancer stem cells' invasive phenotype could be an important step in the treatment of GBM patients.

Rai belongs to the Shc family of adaptor proteins [5–7]. The *RAI* gene encodes for two isoforms, p52Rai and p64Rai, with an identical PTB-CH1-SH2 structure but for the addiction of an N-terminal CH2 domain in p64Rai; however, nothing is known about a possible difference in their role [8]. In the mouse adult brain, Rai is expressed in neurons where, after ischemic damage, it implements a pivotal neuroprotective response by promoting the activation of the PI3K/Akt pathway, which leads to increased cell survival [9, 10]. Notably, while Rai is not normally expressed in glial cells [11], it is ectopically expressed in high-grade gliomas [12] where the PI3K/Akt signaling pathway is frequently activated [13].

To investigate its role in the development of glial tumors, we used the neurosphere (NS) system as a good surrogate for the in vitro study of normal and tumor stem and progenitor cells [14, 15]. NSs are a mixed population of neural stem, progenitor and differentiated cells. Several markers (e.g., CD133 and CD15) have been described for the isolation of pure stem cell population, but no incontrovertible evidence is available for any of them [16, 17]. In this manuscript, we present a series of results that indicate Rai as a novel modulator of neural stem/progenitor migration. Indeed, our in vivo data demonstrate that Rai silencing in GBM NSs provides survival benefit by generating tumors with a poor infiltrative pattern. Rai function in GBM stem/progenitor cells is

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reminiscent of its function in neural stem/progenitor cells; Rai−/− mice show a reduced migration of neural progenitors to the olfactory bulb (OB). Our findings support the potential use of Rai as a target to block brain cancer invasion and could have clinically relevant implications.

Materials and Methods

GBM Specimens

Tumor samples classified as GBM according to the WHO classification were collected from consenting patients undergoing surgery at the Neurosurgery Dpt. IRCCS Istituto Clinico Humanitas. Fresh tumors were processed for the isolation of stem/progenitor cell-containing NSs immediately after surgery. GBMs were cut into small pieces, digested mechanically and enzymatically with papain (Worthington Biochemical, Lakewood, NJ, http://worthington-biochem.com) and filtered with a 70 μm cell strainer [18]. A small part of the tumor was either processed and paraffin embedded for histology or used for WB/quantitative real time polymerase chain reaction (qRT-PCR) analysis.

Ethics Statement

Experiments involving animals were performed in accordance with the Italian Laws (D.L.vo 116/92 and following additions), which enforce EU 86/609 Directive (Council Directive 86/609/EEC of November 24, 1986 on the approximation of laws, regulations, and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes).

Animals

For experiments on normal neural stem/progenitor cells, age- and sex-matched Rai−/− and wild-type (WT) 129SV mice were used. Rai−/− mice are a *Rai-LacZ* knockin mouse model (referred as Rai−/− throughout the whole text), expressing β-galactosidase in place of Rai [19]. For normal NS cultures, the subventricular zone (SVZ) of the brain of 2-month-old female Rai−/− and WT mice was dissected under a microscope (Leica MZ6, Germany, http://www.leica-microsystems.com), mechanically dispersed, and enzymatically digested with papain (Worthington Biochemical, Lakewood, NJ, http://worthington-biochem.com). For ex vivo experiments, mouse SVZ cultures were derived and grown as previously described [20]. For intracranial injections, 10^5 cells from dissociated GBM NSs were resuspended in 2 μl of phosphate buffered saline and stereotaxically injected into the nucleus caudatus of 5-week-old female CD-1 nu/nu mice (Charles River, Wilmington, MA, http://www.criver.com). All transplanted mice were maintained until development of neurologic signs.

In Vivo BrdU Administration

BrdU (75 mg/kg, Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) was dissolved in phosphate buffered saline PBS and intraperitoneally injected in 2-month-old mice for 10 days, followed by 2 days of chase; then the animals were sacrificed and brains were processed for immunofluorescence staining. The experiments were repeated three times and cell count was performed on five to six series of adjacent sections per brain (SVZ, rostral migration stream [RMS], and OB were entirely analyzed by cutting all the brain in serial sections, and the cell counts were performed in the same region of WT and Rai−/− brains).

Cell Culture and Experimental Procedures

Normal and GBM NSs were maintained in NeuroCult medium (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) supplemented with 20 ng/ml epidermal growth factor (EGF), 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, Rocky Hill, NJ, http://www.peprotech.com), and 0.0002% heparin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com).

To induce differentiation, dissociated NSs were plated on Matrigel-coated six-well plates in NeuroCult basal medium supplemented with 10% fetal bovine serum (FBS, Lonza Bio-Whittaker, Basel, Switzerland, http://bio.lonza.com) and cultures were maintained for 7 days. 293T-Rai cells were obtained after the transfection of a pCDNA3.1-*RAI* expression vector [9] and grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Paisley, UK, www.invitrogen.com) supplemented with 10% FBS, penicillin/streptomycin (100 U/ml), and 1% glutamine. GBM NSs expressing the nuclear factor κB (NFκB) p65 subunit were generated by retroviral infection using supernatant from Phoenix amphotropic packaging cells transfected with pBabe-*p65* (gift from Gioacchino Natoli, European Institute of Oncology, Milan, Italy). GBM NSs expressing Notch intracellular domain (NICD) were generated by retroviral infections using supernatant from Phoenix amphotropic packaging cells transfected with pBabe-NICD vector encoding the constitutively active human NOTCH-1ΔE mutant [21]. NFκB reporter assay was performed by transfecting a NFκB luciferase reporter vector and a Renilla luciferase reporter plasmid as internal control. The luciferase assay was measured 48 hours after transfection using a 96-well luminometer with the Dual Luciferase Reporter Assay system (Promega, Madison, WI, http://www.promega.com). The luciferase activity was normalized with the internal control Renilla luciferase.

Lentiviral-Mediated Rai Silencing and Rai Overexpression

Lentiviral vectors for overexpression were obtained from Didier Trono (University of Geneva, Switzerland) and pLentiLox 3.7 was used for *Rai* targeting. Target sequence was: 5′-GGAAAGACCTCTTTGACAT-3′ for *RAI* short hairpin RNA (Rai shRNA) and a scrambled short hairpin was used in the nontargeting control (NT shRNA). Viral infection was performed according to Trono's protocol.

Cell Migration and Invasion Assays

Wound-healing migration assays were performed plating dissociated NSs on Matrigel (BD Biosciences, Franklin Lakes, NJ, http://bdbiosciences.com; 1:100 diluted with DMEM) in NeuroCult basal medium supplemented with FGF only and making a wound with a pipette tip when the cells were confluent. Photographs were taken to monitor cell migration across the wound. Both Boyden chamber migration and invasion assays were performed using Transwell Permeable Supports (Corning Incorporated, Corning, NY, http://www.corning.com). For the sphere dispersion assay, GBM NSs were allowed to adhere on Matrigel-precoated plates and cell dispersion out of the sphere was monitored for up to 24 hours.

Western Immunoblotting

Total protein from both GBM specimens and NS cultures were analyzed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) and Western blotting according to established procedures. Primary antibodies used were: Rai (mouse monoclonal; BD Transduction Laboratories), β-catenin (mouse monoclonal; BD Transduction Laboratories,

http://www.bdbiosciences.com), P∼ β-catenin Thr41Ser45 (rabbit polyclonal; Cell Signaling, http://www.cellsignal.com/), doublecortin (Dcx) (rabbit polyclonal; Abcam, http://www.abcam.com), Notch-Valine 1744 (rabbit polyclonal; Cell Signaling), NFκB p65 subunit (rabbit polyclonal; Cell Signaling), and NFκB P∼ p65 Ser536 (rabbit polyclonal; Cell Signaling). Actin (mouse monoclonal; Sigma-Aldrich) and Vinculin (mouse monoclonal; Sigma-Aldrich) were used to normalize the amount of lysate loaded on the gels. After incubation with an horseradish peroxidase-conjugated-conjugated secondary antibody (Sigma-Aldrich, http://www.sigmaaldrich.com), protein bands were visualized using the SuperSignal West Pico Substrate (Pierce, Rockford, IL, http://www.piercenet.com).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Analysis

RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, http://www.qiagen.com) according to the manufacture's instructions. qRT-PCR was performed using the Sybr Green PCR Master Mix (Applied Biosystems) and signals were detected using a 7500 Fast Real-Time PCR System (Applied Biosystems, US, http://appliedbiosystems.com). Data were analyzed with the AB Sequence Detection Software, Version 1.4 (Applied Biosystems) using *glyceraldehyde 3-phosphate dehydrogenase* as a housekeeping gene. All samples were run in triplicate in each experiment and results were expressed as mean ± SD.

Immunohistochemistry and Immunofluorescence Analysis

For immunohistochemistry, mouse brains and human surgical samples were formalin-fixed and paraffin embedded according to the established procedures. All sections were counterstained with Mayer's hematoxylin and visualized using a bright-field microscope. For immunohistochemistry on paraffin-embedded NSs, fixed GBM NSs were suspended into 1% agarose and embedded in paraffin. Confocal images and live-microscopy images were generated with a Leica TCS SP2 confocal microscope driven by Leica software (Leica Microsystems, Germany, http://www.leica-microsystems.com). The following primary antibodies were used: green fluorescent protein (GFP) (mouse monoclonal; SantaCruz), Rai (mouse monoclonal, immunopurified antibody generated by the Antibody Facility at the IFOM-IEO Campus, Milan, Italy; validation by immunohistochemistry is shown in Supporting Information Fig. S1), Dcx (rabbit polyclonal; Abcam), Olig2 (rabbit polyclonal; Abcam), mitochondrial marker (mouse monoclonal; Abcam), Ki67 (mouse monoclonal; BD Pharmingen), cleaved caspase-3 (rabbit polyclonal; Cell Signaling), BrdU (rat polyclonal; Immunological Direct, AbCys, France, http://www.abcysonline.com), β-catenin antibody (mouse monoclonal; BD Transduction Laboratories), and anti-human nuclei (mouse monoclonal; Millipore, Billerica, MA, http://www.millipore.com).

For tumor area quantification, each brain was sliced into several coronal sections and the sections with the largest tumor diameter were considered as corresponding to the highest degree of tumor invasion [22]. These sections were immunostained with the anti-human nucleus antibody to clearly identify infiltrating human tumor cells; the tumor area was measured with ImageJ free software (rsbweb.nih.gov/ij/).

Statistical Analysis

Statistical analyses were carried out using the SPSS software package (Statistical Package for the Social Sciences, http://www.ibm.com). Data graphed with error bars represent mean ± SD. Two-sided Student's *t* test or one-way analysis of variance was used to determine the significance of any differences between experimental groups. For the in vivo limiting dilution assay, tumor formation frequency and statistical significance were evaluated with the extreme limiting dilution analysis function (http://bioinf.wehi.edu.au/software/elda/). MedCalc software (http://www.medcalc.org) was used to create Kaplan Meyer curves and compare survival with log-rank analysis. Differences were considered significant when $p < .05$ (*) and highly significant when $p < .01$ (**).

Results

Rai Is Highly Expressed in GBM NSs

Ectopic Rai expression has been detected in human high-grade astrocytomas (anaplastic astrocytomas, WHO grade III, and GBMs, WHO grade IV) [12]. We have thus performed a detailed immunohistochemistry analysis of whole sections of human GBMs and found that Rai was expressed in all the samples analyzed but its distribution inside the tumor was not homogeneous (Supporting Information Table S1). In some samples, only few isolated Rai-positive cells, surrounded by tumor tissue, were detectable, whereas in others Rai expression seemed to be zonal. Interestingly, in all the cases, intense Rai staining was detected at the tumor/host interface, as shown in Figure 1A for GBM#14. We established NS cultures from the tumors in Table S1 by cultivating the cells under standard neural stem cell culture conditions and assessing their expression of the putative stem cell marker CD133 and their in vivo tumor propagation (Supporting Information Table S2). Regardless of Rai expression in the primitive tumor, and even when Rai-positive cells were rare, Rai was always similarly upregulated in all GBM NSs, suggesting that it was expressed in GBM undifferentiated cells (stem cells and/or progenitors making up most of the NS) (Fig. 1B). Similarly, at RNA level, *RAI* was almost undetectable in patient tumors but highly expressed in the matching GBM NSs, like the putative glioma stem/progenitor cell marker *OLIG2* [16] (Fig. 1C, 1D). In order to exclude possible culture artifacts affecting Rai expression, we also studied Rai expression in tumor NSs cultured without EGF and FGF; however, Rai expression did not appear significantly different in these conditions (Supporting Information Fig. S2). Immunohistochemistry on paraffin-embedded NSs revealed that Rai was expressed in the majority of the cells (Fig. 1E). When CD133-positive and negative fractions were directly isolated from either patient specimens or short-term NS cultures, both subpopulations equally expressed Rai (data not shown). Interestingly differentiation of the NSs induced a marked decrease in Rai expression (Fig. 1F). These data suggest that Rai is expressed in the main population (>90%) of cells inside GBM NSs, likely stem/progenitor cells.

Rai Silencing in GBM NSs Increases Tumor Latency

To unravel the role of Rai in gliomagenesis, we silenced Rai expression in GBM NSs by cloning shRNA oligonucleotides specific for human *RAI* cDNA into a lentiviral vector containing the *GFP* and a *puromycin resistance* marker. Since Rai was expressed at similar levels in all GBM NSs, regardless of the type of expression in the parental tumor, we transduced NSs derived from tumors showing different patterns of Rai distribution, that is, tumors #7, #10, #18, and #20 expressing high levels of Rai protein, tumor #8 and #11 with low Rai expression levels, and tumors #9 and #22 with moderate Rai

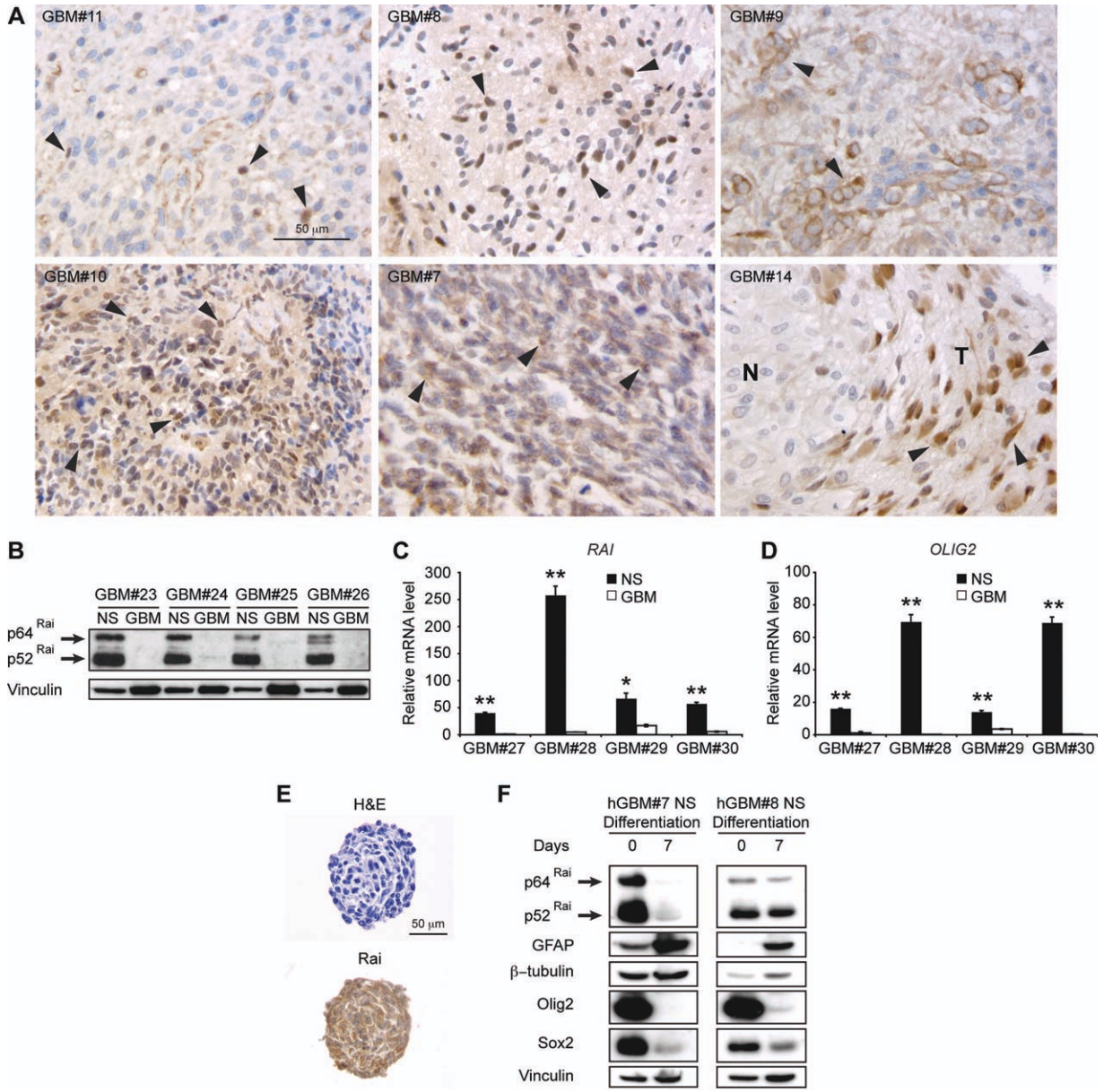

Figure 1. Rai, heterogeneously expressed in hGBMs, is always enriched in GBM stem/progenitor cells. **(A):** Immunohistochemical staining for Rai in hGBM surgical biopsies: heterogeneous Rai expression among different samples (GBM#11, GBM#8, GBM#9, GBM#10, GBM#7 and GBM#14). Images representative of 22 patients analyzed. Note the strong expression of Rai at the tumor/host interface (GBM#14: N, normal brain; T, tumor). Representative Rai-positive cells are indicated (arrowheads). **(B):** Western blotting: Rai expression is higher in GBM NSs compared with matched parental fresh tumors (GBM). **(C, D):** Quantitative real time polymerase chain reaction: *RAI* and the putative stem/progenitor cell marker *OLIG2* are highly expressed in GBM NSs compared with matched parental fresh tumors (GBM); mean ± SD, *, $p < .05$ and **, $p < .01$. **(E):** Immunohistochemistry on paraffin-embedded GBM NSs: Rai is expressed by nearly all cells inside the NS. **(F):** Western blotting: Rai expression decreases in differentiating GBM NSs. Note also the decreased expression of Olig2 and Sox2 (markers of undifferentiated cells) and increased expression of differentiation markers (GFAP and β-tubulin III). Abbreviations: GFAP, glial fibrillary acidic protein; hGBM, human glioblastoma; NSs, neurospheres.

expression (Supporting Information Table S1). Interference efficiency was confirmed by Western blot: the protein was silenced by nearly 90% in comparison to its expression in nontransduced glioma cells (naïve cells) and nontargeted control cells (NT shRNA) in all samples (Fig. 2A and Supporting Information Fig. S3A). In accordance with protein results, *RAI* expression was reduced at the mRNA level as well (Fig. 2B).

To determine the in vivo relevance of Rai silencing, we assessed tumor initiation and propagation by performing an orthotopic transplantation assay. We stereotaxically implanted naïve cells and glioma cells infected with a lentivirus expressing either NT shRNA or Rai shRNA into the nucleus caudatus of immunodeficient mice. All animals transplanted with naïve and control cells developed neurological signs and died due to the presence of large intracranial tumors. Injection of Rai-silenced cells delayed the onset of neurological signs and significantly increased mice survival (Fig. 2C and Supporting Information Fig. S3B). Rai interference, therefore, reduces the tumorigenic potential of GBM NSs, suggesting that Rai function in GBM stem/progenitor cells is critical for tumorigenesis.

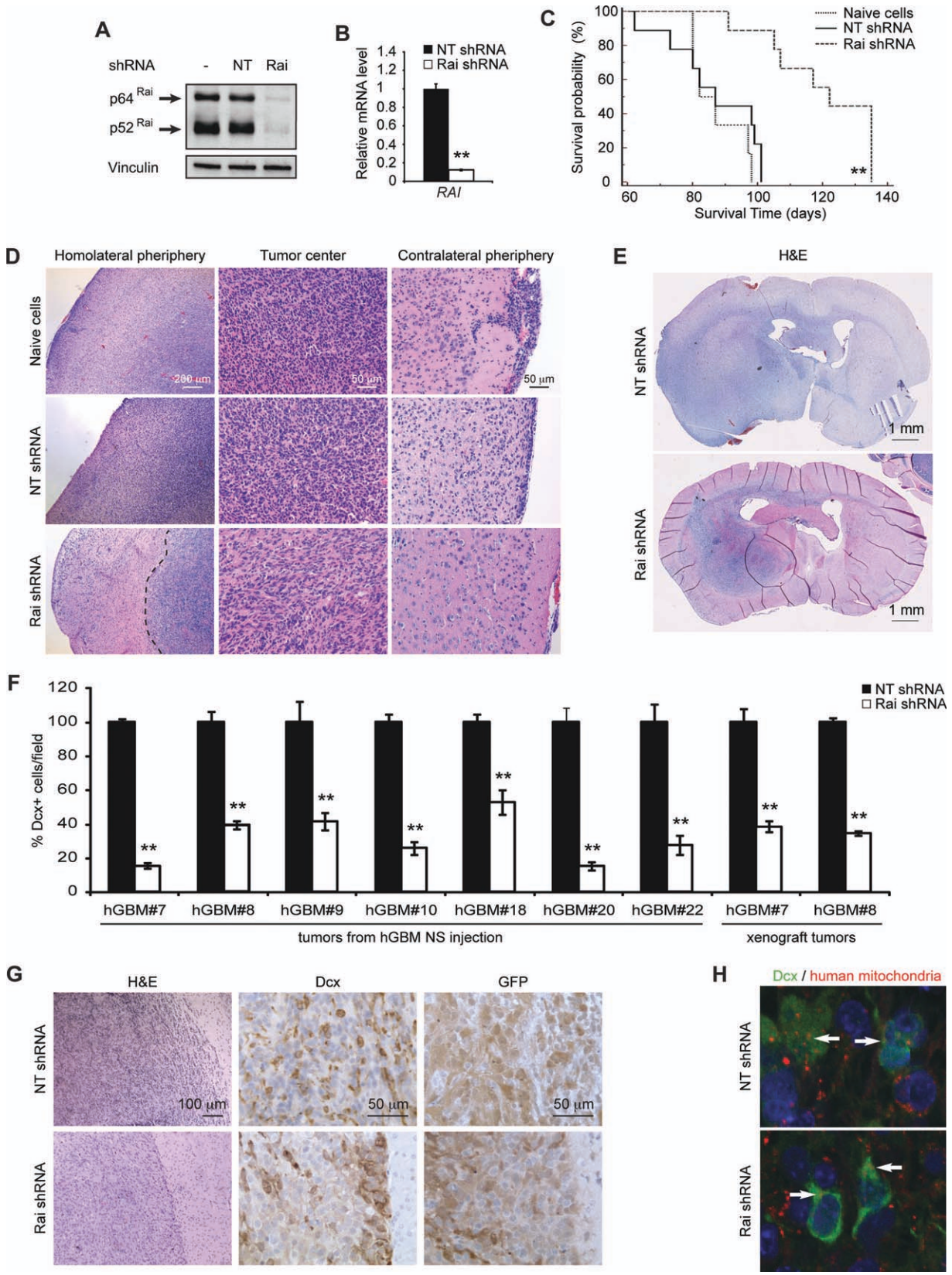

Figure 2.

Rai Silencing Reduces Tumor Invasion In Vivo

Histological examination of brain sections revealed that after injection of control NSs, but not of Rai-silenced ones, extensive lesions had formed, with a higher cellular density in the center of the tumor and diffuse infiltration in the surrounding brain. The presence of tumor masses in both the homolateral and contralateral hemispheres was a clear evidence of the invasive nature of the control tumors. In contrast, tumors derived from Rai-silenced cells had more demarcated borders, reduced cellularity in the center of the tumor mass, and less infiltrative tumor cells in both the homolateral and contralateral hemispheres (Fig. 2D, 2E and Supporting Information Figs. S3C, S4). We also measured the maximal tumor areas per coronal section [22] confirming that the areas of Rai-silenced tumors were significantly reduced compared with control tumors (Supporting Information Fig. S4). However, we did not find any significant difference between Rai-silenced and control tumors, either in the proliferative index, measured as percentage of Ki67 positive cells (Supporting Information Fig. S5), or in vascularization and necrosis degree (data not shown). Importantly, a significant reduction in the number of tumor invading cells, measured as Dcx-positive cells, was found in all Rai-silenced tumors examined (Fig. 2F). Dcx is a marker of migrating neuroblasts during development and in the adult normal brain, and it is a marker of infiltrating cells in GBMs [23, 24]. These Dcx-positive cells were positive for GFP and for the human mitochondrial protein [25], demonstrating their identity as human tumor cells (Fig. 2G, 2H and Supporting Information Fig. S6).

Rai distribution in the control xenografts recapitulated the expression pattern of the parental tumors: only scattered cells expressed Rai in the control xeno-tumors (Fig. 3A) when the NSs (hGBM#8 NS) had been isolated from human GBMs with similar pattern of Rai expression (GBM#8 in Fig. 1A). In control tumors, Rai-positive cells were found at the tumor center and tumor periphery. As expected, Rai was barely expressed in the center of tumors derived from Rai-interfered cells. Surprisingly, in all the samples examined, most of the rare cells still expressing Rai were localized at the tumor invasion front, thus suggesting a role for Rai in tumor invasion (Fig. 3A). A similar expression pattern was also found in all Rai-silenced tumors: Rai was undetectable in the tumor center, and the only Rai-positive cells were localized at the tumor edge (Supporting Information Fig. S7).

By analyzing serial sections, we found that Rai-positive cells also expressed GFP, confirming their derivation from the injected human tumor cells (Fig. 3B). Moreover, Dcx and Olig2 immunostaining paralleled Rai expression in all the tumor sections analyzed (Fig. 3B). Olig2 is a neural stem/progenitor transcription factor that regulates NS formation in vitro and glioma formation in vivo [16]. These findings suggest that the rare Rai-positive cells in Rai-silenced xenografts correspond to immature and highly migratory cells responsible for GBM invasion. When Rai was not completely knocked-down (Supporting Information Fig. S8A), the mice developed diffusively infiltrating gliomas with latencies similar to those of the controls (Supporting Information Fig. S8B, S8C).

We also performed a serial transplantation assay. Tumors from hGBM#7 and hGBM#8 xenotransplanted mice were collected and used to establish secondary GBM NS cultures; Rai silencing was maintained in both the hGBM#7 and hGBM#8 Rai shRNA cells (Supporting Information Fig. S9A). We next implanted naïve, control, and Rai-interfered cells in immunocompromised mice: Rai targeting delayed tumor expansion, significantly increasing the lifespan of the injected mice (Supporting Information Fig. S9B, S9C). These results confirm the in vivo survival benefit as a consequence of Rai knockdown, as shown in the primary xenotransplants.

The secondary xenografts initiated by Rai shRNA NSs possessed the same characteristics of the primary transplanted tumors from which they had been isolated; they maintained distinct boundaries (Supporting Information Fig. S9D) with less-disseminated Dcx-positive cells (Fig. 2F), indicative of a reduced infiltrative growth. Moreover, they retained Rai downregulation, with the exception of rare cells at the tumor edge (Supporting Information Fig. S9D). These results reinforce the concept that Rai acts as a modulator of GBM invasion into the surrounding normal brain.

To investigate whether the reduced tumorigenic potential of Rai-interfered cells could be the consequence of a lower number of cancer stem cells, we performed an in vivo limiting dilution assay. Equal cell numbers from dissociated control and Rai-interfered NSs were injected into nude mice at four different doses (10^2–10^5) to assess tumor growth. The frequency of tumor growth observed per number of transplanted cells is an indication of the frequency of tumor-initiating cells [26]. Since control and Rai-silenced groups developed tumors with similar incidence in all the cases studied (Supporting Information Table S3), Rai silencing does not appear to affect cancer stem cell frequency. However, mice survival was significantly increased in the absence of Rai at each cellular dose tested in all the cases studied (Supporting Information Table S3).

Rai Silencing Reduces In Vitro Invasion and Migration Ability of GBM Stem/Progenitor Cells

We then studied Rai's contribution to in vitro cell migration and infiltration of GBM NSs. Rai's downregulation in NSs

Figure 2. Rai silencing in GBM NSs results in improved mice survival and reduced GBM invasiveness. **(A, B):** Rai silencing efficiency in GBM NSs from hGBM#8 patient as assessed by Western blot (A) and quantitative real time polymerase chain reaction (B); mean ± SD, **, $p < .01$. NT: nontargeting shRNA. **(C):** Kaplan-Meyer survival curve: increased mice survival after injection of Rai-silenced cells into the nucleus caudatus of immunodeficient mice; **, $p = .0023$ for 10^5 injected hGBM#8 cells; $n = 6$ mice for naïve cells, $n = 9$ mice for NT shRNA cells, $n = 9$ mice for Rai shRNA cells. **(D):** H&E staining of a representative xenograft tumor derived from naïve, NT shRNA and Rai shRNA hGBM#8 cells (three mice per group were analyzed). Homolateral hemispheres, tumor center, and contralateral hemispheres are shown. Note the invasive tumor cells localized away from the main tumor mass, infiltrating the homolateral and the contralateral periphery in control brains, and the smoother borders of Rai-silenced tumors, which could be easily demarcated (dashed line). **(E):** H&E staining of representative brain sections from mice injected with NT shRNA and Rai shRNA hGBM#8 cells. Rai-silenced tumors are more circumscribed compared with control tumors, which display widespread invasion of the surrounding brain. **(F):** Quantification of invading Dcx-positive cells outside the tumor core; mean ± SD, **, $p < .001$. The counts were performed at the invasive front of the tumor ($n = 3$ mice; three sections per mouse were analyzed). **(G):** H&E staining and immunostaining for Dcx and GFP on a representative xenograft tumor, derived from NT shRNA and Rai shRNA hGBM#22 cells. The GFP protein, encoded by the lentiviral transgene, identifies human tumor cells. **(H):** Confocal analysis of immunofluorescence staining for Dcx (green) and human mitochondria (red) on representative xenograft tumors derived from NT shRNA and Rai shRNA hGBM#22 cells. Nuclei were counterstained with DAPI (blue). Human mitochondria immunoreactivity identifies human tumor cells. Arrows point to double positive cells (Supporting Information Figs. S3, S4, S6). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; Dcx, doublecortin; GFP, green fluorescent protein; hGBM, human glioblastoma; NSs, neurospheres; NT shRNA, nontargeting short hairpin RNA.

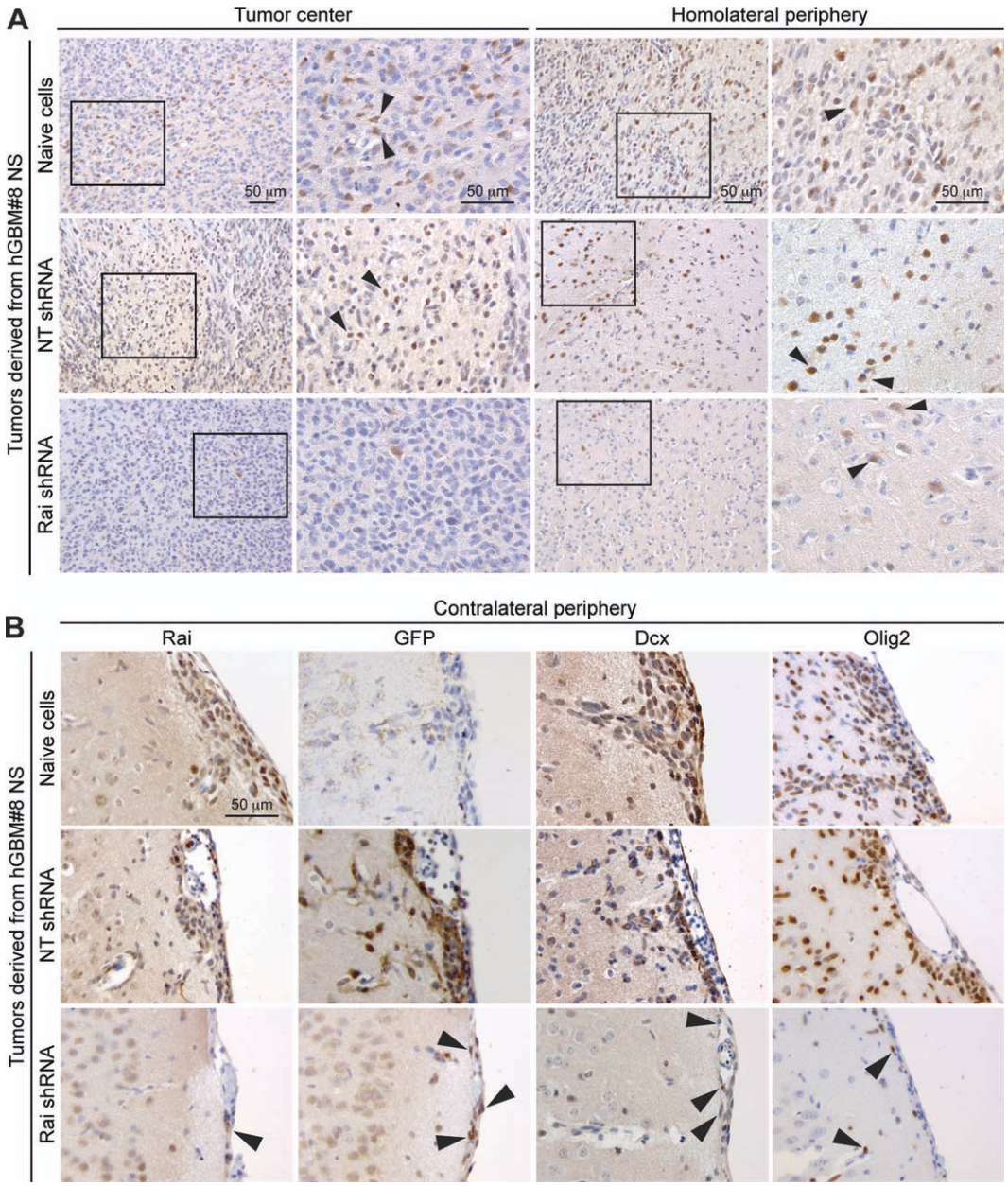

Figure 3. Rai is distributed at the periphery of the tumor. **(A):** Representative immunohistochemical staining for Rai in controls and Rai-silenced xenograft tumors derived from hGBM#8 cells. For each group, representative images from the tumor center and homolateral periphery are displayed. In controls, Rai-positive cells (arrowheads) are found in both tumor center and tumor edge. In Rai-silenced groups, no staining is found in the tumor center; there are rare Rai-retaining cells (arrowhead) only in the tumor infiltration zone. **(B):** Rai, GFP, doublecortin (Dcx), and Olig2 staining on adjacent sections revealing tumor cells infiltrating the contralateral hemisphere and reaching the cortex. Rai-positive cells express GFP, the migratory marker Dcx, and the putative stem/progenitor cell marker Olig2 both in controls and Rai-silenced groups. Note the decreased number of Rai-positive cells (arrowheads) in the contralateral periphery of mice bearing Rai-silenced tumors (Supporting Information Fig. S7). Abbreviations: GFP, green fluorescent protein; hGBM, human glioblastoma; NSs, neurospheres; NT shRNA, nontargeting short hairpin RNA.

derived from several patient samples and xenotransplanted mice decreased cell infiltration through the Matrigel-coated membranes of nearly 50% (Fig. 4A). To further confirm the role of Rai in cell invasion, GBM NSs were allowed to adhere and spread three dimensionally in Matrigel. NS dispersion was monitored by time-lapse imaging: 4 hours after plating, in the absence of Rai, only few cells had migrated away from the NSs; in contrast, control NSs were clearly more dispersed in Matrigel (Fig. 4B). Rai depletion significantly decreased cell dispersion of the NSs studied (Fig. 4C). Since cell motility contributes to the invasive phenotype of malignant glioma, we verified whether Rai affects cell migration. Control cells from patient tumors displayed significant mobility in a wound-healing assay, even if at different migration rates depending on the sample. On the contrary, none or few Rai-interfered cells migrated in the wound (Fig. 4D). The Boyden chamber assay confirmed the reduced glioma cell migration in the absence of Rai in all the cases studied (Fig. 4E). These invasion and migration defects were not dependent on the reduced cell proliferation of Rai-interfered cells (data

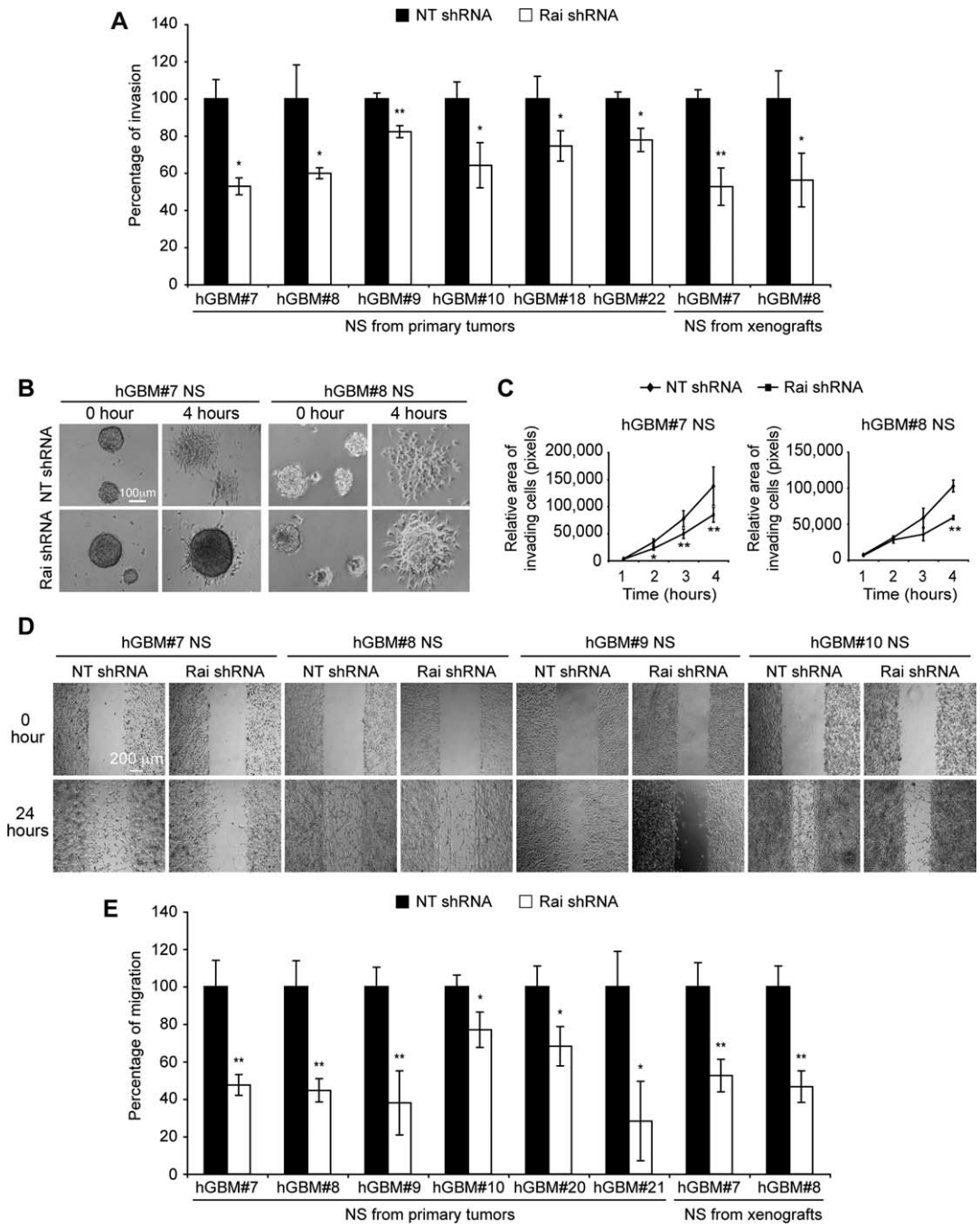

Figure 4. Rai silencing impairs migration and invasion of GBM stem/progenitor cells in vitro. **(A):** Quantification of invading cells with Boyden chamber invasion assay: in the absence of Rai, in vitro invasiveness is decreased in all NSs tested, derived either from human parental tumors or xenografts; mean ± SD, *, $p < .01$ and **, $p < .001$. **(B, C):** Cell dispersion from hGBM#7 and hGBM#8 Matrigel-embedded NSs is impaired when Rai is silenced. Images shown in (B) are representative of three independent experiments. (C) Quantification of cell dispersion from hGBM#7 and hGBM#8 NSs at the indicated time points. The distance from the edge of each NS to the most distal migrating cell is expressed as relative area of invading cells: hGBM#7: 1 hour, *, $p = .045$, 2 hours, **,$p \ll .001$, 3 hours, **, $p = .001$, 4 hours, **, $p = .003$; hGBM#8: 3 hours, $p = .052$, 4 hours, **, $p \ll .001$; mean ± SD. **(D):** Wound-healing assay: Rai silencing reduces cell migration. Images shown are representative of three independent experiments. **(E):** Quantification of migrating cells with Boyden chamber migration assay: Rai-silenced cells show reduced migration ability compared with control cells; mean ± SD, *, $p < .01$ and **, $p < .001$. Abbreviations: hGBM, human glioblastoma; NSs, neurospheres; NT shRNA, nontargeting short hairpin RNA.

not shown). Taken together, our findings of decreased in vitro cell migration and invasion correlate well with the reduced level of infiltration in Rai-silenced xenografts, suggesting that loss of Rai expression affects the migratory and invasive responses of GBM NS cells.

Rai Deletion Causes Migration Defects in Normal Adult Brain

We asked whether Rai, expressed only in mature neurons in the adult brain [11], was also expressed in normal neural

stem/progenitors cells, which are the putative GBM cells of origin [27]. Immunohistochemistry and X-gal staining on adult brain sections revealed Rai expression in neuroblasts along the RMS (Fig. 5A and 5B) and in some cells within the SVZ (Fig. 5C and Supporting Information S10A).

Further evidence of Rai functional relevance in the neural stem/progenitor cells was provided by the detection of Rai mRNA and protein in the NSs derived from adult SVZ (Fig. 5D and 5E). A marked increase of Rai expression was observed in cultures enriched for progenitor cells at both mRNA and protein level (Fig. 5D and 5E). Rai was homogeneously distributed in the stem/progenitor cells composing the sphere (Fig. 5F), mirroring the widespread expression of Rai observed in GBM NSs (Fig. 1E).

Neural progenitor migration continuously occurs in the adult rodent brain from the SVZ, where neural stem cells

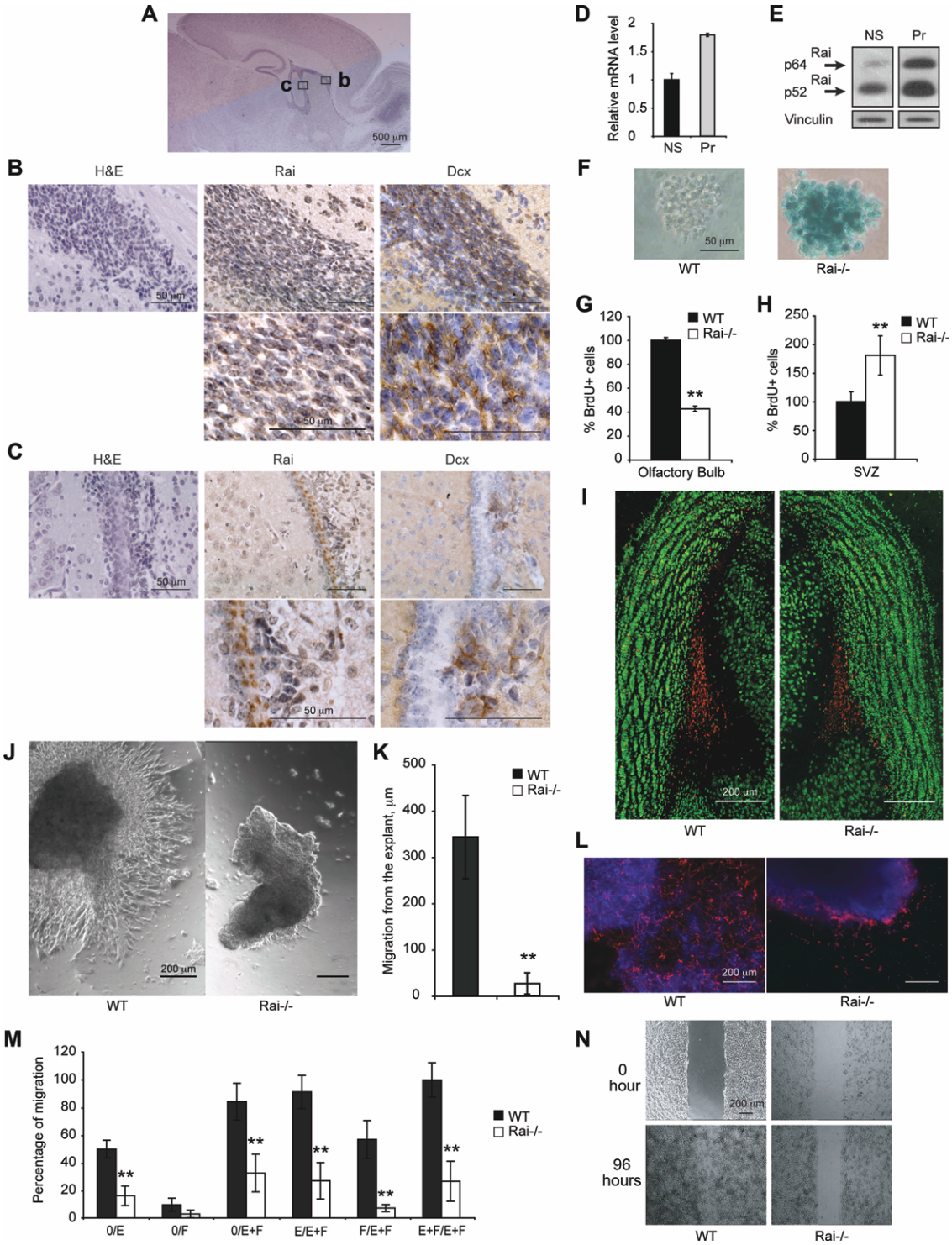

Figure 5.

reside, to the OB, where neural progenitors are integrated into the existing structures and replace neurons [28, 29]. We administered BrdU to WT and Rai−/− mice. In Rai−/− mice, we found a 60% reduction of BrdU-positive cells within the OB (Fig. 5G and 5I); this reduction was not due to a proliferation defect, since a higher number of BrdU-positive cells was present in the SVZ of Rai−/− brain than that of WT brain, possibly reflecting accumulation of progenitors in this neurogenic area due to a migration impairment (Fig. 5H). Indeed, immunostaining for Ki67 and cleaved caspase-3 revealed that in the SVZ of WT and Rai−/− brains, there were no differences either in the number of proliferating cells (Supporting Information Fig. S10B, S10C) nor in the apoptosis rate (Supporting Information Fig. S10D). These data provide evidences that Rai contributes to the migration of neural progenitors from the SVZ to the OB.

We then cultured SVZ explants embedded in Matrigel and measured the ability of neuroblasts to migrate outside the explants: neuroblast migration from Rai−/− SVZ explants was severely affected, resulting in a 90% reduction in migration distance (Fig. 5J, 5K). The migrating neuroblasts were positive for Dcx (Fig. 5L). The migration ability of SVZ-derived NS cells was analyzed in vitro as well. We performed a Boyden chamber assay either under standard culture conditions or in the presence of different combinations of the two growth factors EGF and FGF as attractors. In all tested conditions, Rai−/− cells displayed a markedly impaired migration capacity (Fig. 5M). A wound-healing assay was performed on a confluent monolayer of progenitors; in contrast with WT cells, Rai−/− cells were not able to migrate across and fill in the wound (Fig. 5N).

Rai Silencing Reduces the Levels of Proteins Involved in Migration/Invasion

Different types of matrix metalloproteinases (MMPs), known to be highly expressed and active in human gliomas and other invasive tumors [30], were downregulated in Rai-silenced NSs isolated from different patients (Fig. 6A). In particular, by quantitative real time polymerase chain reaction (qRT-PCR) analysis, we found downregulation of *MMP2* and *MMP3*. Expression of *MMP14*, a cell-surface protease that acts not only as an activator of pro-MMP2 but also as an extracellular matrix (ECM) degradation enzyme, was reduced too. Consistent with these findings, tissue *inhibitor of metalloproteinase 3* (*TIMP3*) was significantly upregulated in Rai-silenced NSs compared with control cells. We also found downregulation of different members of both *ADAMs* (a disintegrin and metalloproteinases) and *ADAM with thrombospondin motifs families* (Fig. 6A), also known to be involved in cell migration [31]. Similarly to what we had found in the tumor NSs, we observed a reduction in the mRNA levels of several *ADAMs* and *MMPs* and an increase in *TIMP3* mRNA level in Rai−/− normal progenitor cells (Fig. 6A). Rai silencing was also accompanied by a significant reduction in Dcx expression at the protein level in GBM NSs and in both Rai−/− NSs and Rai−/− progenitor-enriched cultures (Fig. 6B). Re-expression of Rai in tumor-silenced cells was able to restore Dcx expression levels (Fig. 6C) and their migration ability (Fig. 6D). Similarly, re-expression of Rai in Rai−/− normal NSs restored Dcx expression (Fig. 6E) and brought their migration ability back to WT levels (Fig. 6F).

Rai Silencing Impairs Different Signaling Pathways in Normal Stem/Progenitor Cells and in Tumor Stem/Progenitor Cells Derived from Different Patients

In every Rai-silenced GBM NS tested and in Rai−/− progenitors, we observed lower expression of several metalloproteases (Fig. 6A) known to be involved in cell invasion through various signaling pathways, such as PI3K/Akt, Wnt, NFκB and Notch pathways. Rai's nature of adaptor protein led us to analyze its potential role in the regulation of these different pathways. Given the ability of Rai to signal on Akt pathway in mature neurons, we first investigated Akt activation, but we did not find any significant downregulation of this pathway in Rai-silenced cancer cells and in Rai−/− normal NSs (Supporting Information Fig. S11A, S11B), suggesting that Rai does not function as an upstream activator of the PI3K/Akt pathway in normal and cancer neural stem/progenitor cells. Indeed Rai silencing also reduced migration and invasion of cells where the PI3K/Akt pathway was not constitutively activated (hGBM#7 NS and hGBM#22 NS in Supporting Information Fig. S11A), further excluding a role of this pathway in the Rai-induced phenotype. We analyzed the datasets derived from the gliomas of Phillips' study [32] and we found no association between expression levels of *Rai* mRNA and phospho-Akt, as measured by immunohistochemistry (IHC) (Supporting Information Fig. S11C). Both *EGFR* amplification and *PTEN* deletion are well-known alterations that activate Akt; we also found no association between these two genetic alterations and the level of *Rai* expression (Supporting Information Fig. S11C). These results show that the function of Rai in GBM is independent of Akt signaling, unlike its function in mature neurons. Among different signaling pathways involved in the regulation of cell migration, we

Figure 5. Rai is expressed in the neural progenitor cells of the rostral migration stream (RMS) in the adult mouse brain. **(A):** H&E staining on a sagittal section of 9-day-old mouse brain; black boxes indicate RMS and SVZ regions in **(B)** and in **(C)**, respectively. (B, C): Immunohistochemical staining using anti-Rai and anti-Dcx antibodies on adjacent sections: Rai has the same expression pattern of Dcx-positive neuroblasts in the RMS (B) and the SVZ (C). Different magnifications are provided. Scale bar = 50 μm. **(D, E):** Rai is expressed in SVZ-derived NSs and it increases in Pr, as assessed by quantitative real time polymerase chain reaction (D) and Western blot (E) analysis; mean ± SD. **(F):** X-gal staining of NSs: $Rai^{\beta\text{-gal}}$ is homogeneously expressed. **(G–I):** In vivo migration of neural Pr from the SVZ to the olfactory bulb (OB) is impaired in Rai−/− mice, as assessed by cell count of BrdU-positive cells in the two regions. (G): OB: $n = 18$ sections (six per brain, three mice), **, $p < .01$. (H) SVZ: $n = 15$ sections (five per brain, three mice), **, $p < .01$; mean ± SD. (I): Representative confocal image of WT (left) and Rai−/− (right) OB. BrdU-positive cells are in red. NeuN staining of mature neurons was used to highlight OB architecture (green). **(J–N):** Neuroblast migration from Rai−/− SVZ explants is impaired. (J): Representative photomicrographs of SVZ explants from 5-day-old mouse brains embedded in Matrigel. (K): Quantification of average neuroblast migration out of SVZ explants; mean ± SD, $n = 48$ (eight SVZ explants per mouse, six mice), **, $p \ll .001$. (L): Confocal immunofluorescence analysis for Dcx-positive neuroblasts (red) in SVZ explants from WT and Rai−/− mice. Nuclei are counterstained with DAPI (blue). **(M):** Boyden chamber assay: impaired migration ability of Rai−/− progenitor cells compared with WT cells, using different combinations of the two growth factors epidermal growth factor (EGF) and fibroblast growth factor (FGF) as attractors. (0: basal medium without growth factors; E: basal medium plus EGF; F: basal medium plus FGF; E+F: basal medium plus EGF and FGF). 0/E: **, $p = .004$; 0/F: $p = .1$; 0/E+F: **, $p = .009$; E/E+F **, $p = .003$; F/E + F **, $p = .004$; E+F/E+F: **, $p = .003$; mean ± SD. **(N):** Wound-healing assay: impaired migration ability of Rai−/− progenitor cells. Images shown are representative of three independent experiments (Supporting Information Fig. S10). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; Dcx, doublecortin; NSs, neurospheres; Pr, progenitors; SVZ, subventricular zone; WT, wild type.

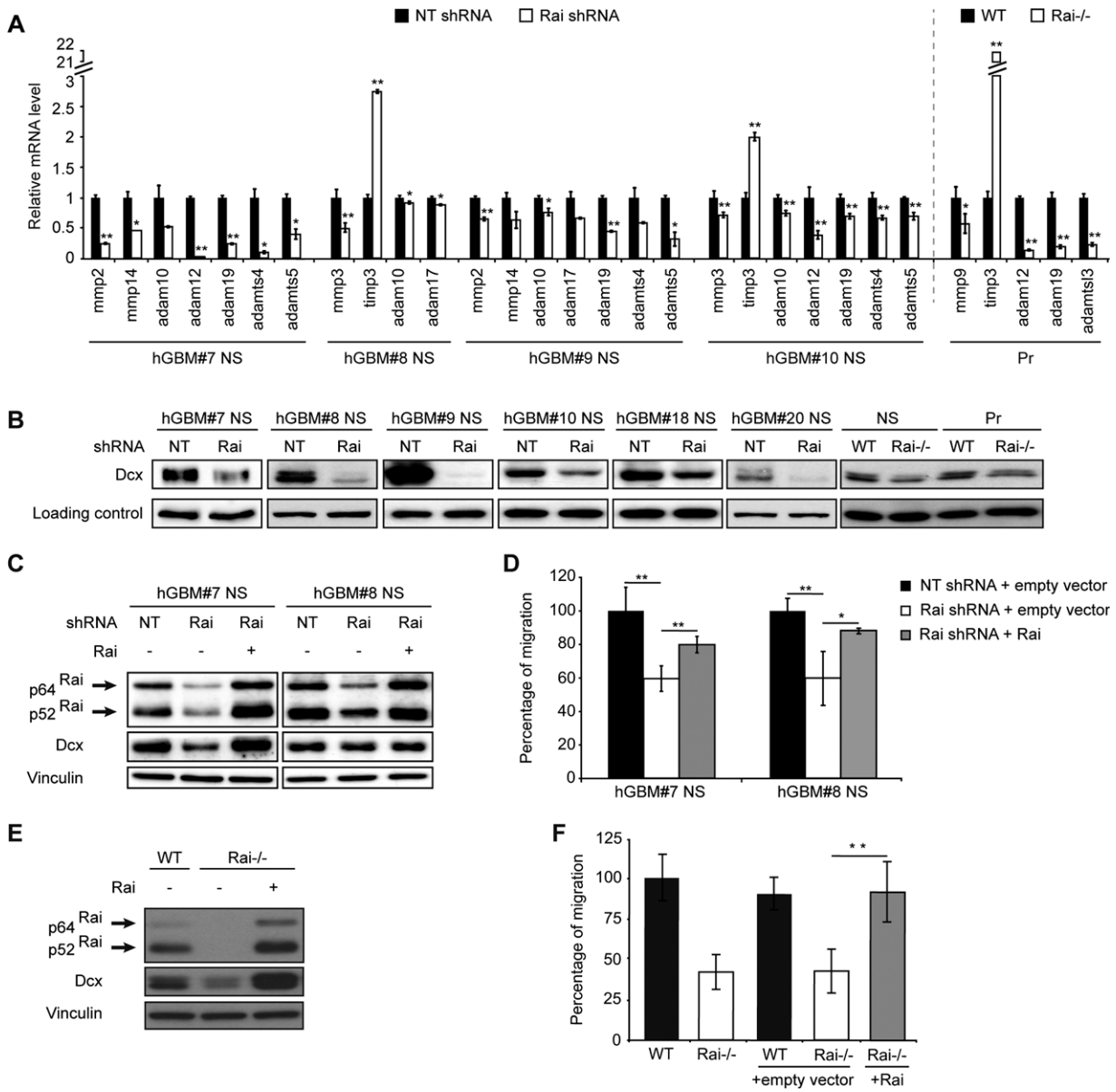

Figure 6. Rai silencing reduces the levels of genes and proteins involved in migration/invasion. **(A):** Quantitative real time polymerase chain reaction (RT-PCR): reduced expression of different proinvasive genes in Rai-silenced GBM NSs derived from different patients and normal murine Pr; mean ± SD, *, $p < .05$ and **, $p < .01$. **(B):** Western blotting: Dcx expression is reduced both in Rai-silenced GBM NSs and in Rai−/− murine NSs and Pr. **(C):** Western blotting: Dcx protein levels are restored in Rai-silenced NSs transduced with a lentiviral vector overexpressing *RAI* cDNA. The empty vector was used as control. **(D):** Reintroduction of Rai in Rai-silenced NSs restores their migration ability; mean ± SD, *, $p < .05$ and **, $p < .01$. **(E):** Western blotting: Dcx protein levels are restored in Rai−/− NSs transduced with a lentiviral vector carrying *RAI* cDNA. The empty vector was used as control. **(F):** Overexpression of Rai in Rai−/− NSs significantly increases their migration ability to levels similar to noninfected and empty vector-transduced NSs. **, $p \ll .01$, one-way ANOVA; Bonferroni multiple *t* test highlights that Rai re-expressing cells migrate significantly more than Rai−/− control cells (**, $p \ll .01$ vs. both Rai−/− cells and Rai−/− mock-transduced cells), reaching an average migration rate consistent with WT cells (naïve and mock-transduced cells) (averages homogeneous as defined by the Tuckey's test, with a $p = .766$); mean ± SD. Abbreviations: Dcx, doublecortin; hGBM, human glioblastoma; NSs, neurospheres; NT shRNA, nontargeting short hairpin RNA; Pr, progenitors; SVZ, subventricular zone; WT, wild type.

found that Wnt/β-catenin pathway was downregulated in normal Rai−/− NSs but not in tumoral NSs. The central effector of this pathway is β-catenin, which, when dephosphorylated, enters the nucleus and activates a number of target genes. β-Catenin was highly phosphorylated in Rai−/− NSs compared with WT cells (Fig. 7A), suggesting that in the absence of Rai β-catenin is less active. Confocal analysis of dissociated NSs showed no β-catenin nuclear translocation in Rai−/− cells; this, in contrast, was well evident in WT cells, in particular after activation of Wnt signaling (Fig. 7B). Moreover, by quantitative real time polymerase chain reaction, we found that the proneural transcription factor *Neurogenin2* (*NGN2*), a regulator of migration and differentiation of normal neural stem/progenitor cells [33] and a known target of β-catenin [34], was strongly downregulated in Rai−/− NSs (Fig. 7C). Consistently, two downstream effectors of *NGN2*, *Neurogenic Differentiation 1 (NEUROD1)*, and *Early B-cell Factor 3 (EBF3)* [35, 36] were drastically downregulated in Rai−/−

NSs (Fig. 7C). However, we observed no differences in the expression of β-catenin target genes involved in proliferation between WT and Rai−/− cells (data not shown).

Considering that Wnt/β-catenin pathway was not altered in Rai-interfered tumoral NSs (Supporting Information Fig. S11D), we studied other signaling pathways. Metalloproteases are targets of the NFκB [37], thus we investigated the influence of Rai on the activation of the NFκB pathway. Surprisingly, we found reduced phosphorylation of the Serine-residue 536 of the NFκB p65 subunit, which in turn indicates reduced

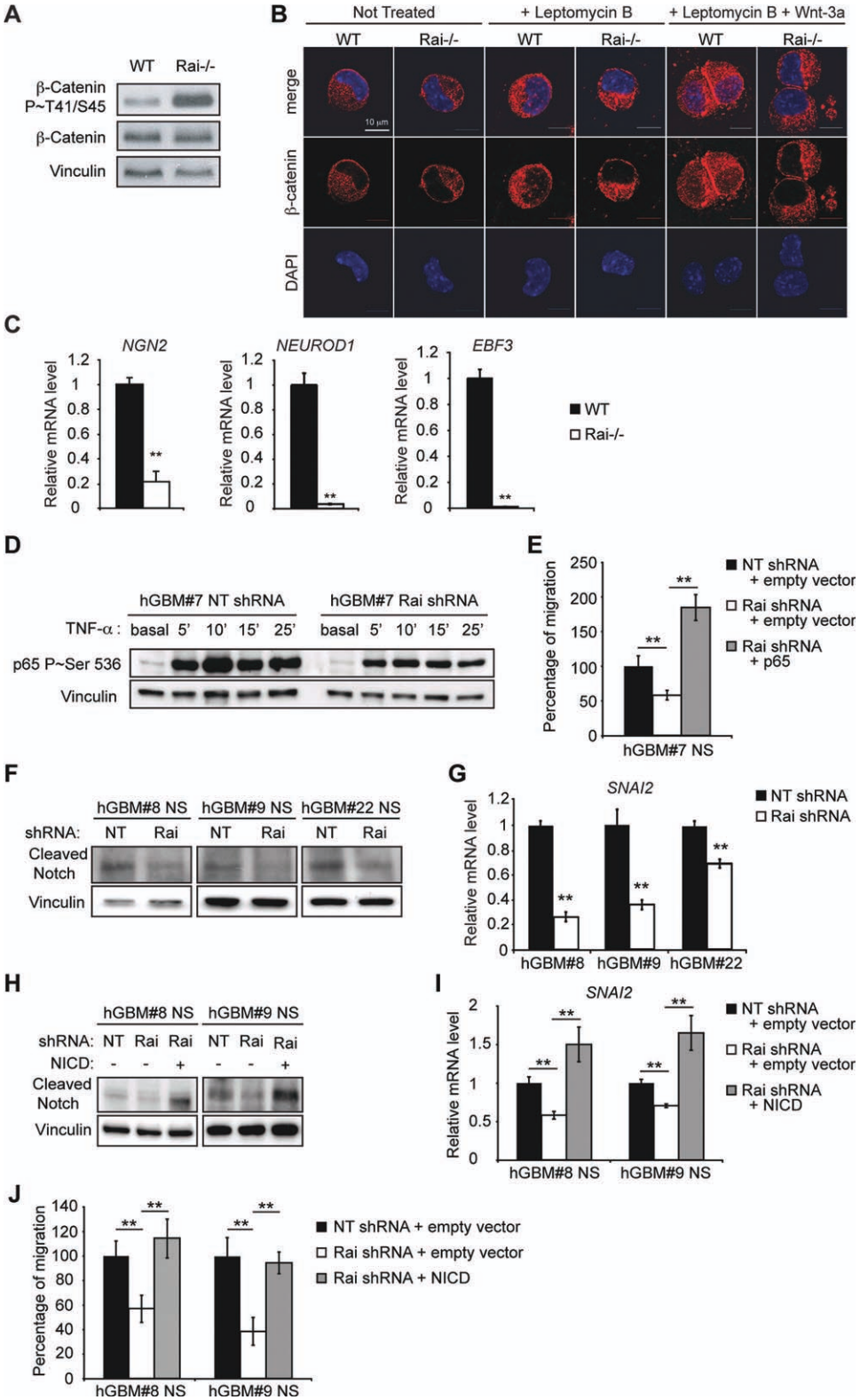

Figure 7.

activation of the NFκB pathway, only in Rai-interfered NSs derived from one patient out of eight analyzed (patient hGBM#7, Fig. 7D). Reactivation of NFkB signaling in these Rai-silenced NSs (Supporting Information Fig. S11E) fully reverted the migration defect (Fig. 7E). To further examine the effect of Rai on NFκB signaling activation, we also overexpressed Rai in the T98G GBM cell line and analyzed nuclear translocation of the p65 subunit, which reflects NFκB pathway activation. Rai overexpression induced a marked increase of p65 in the nuclear fraction after 30 minutes and up to 2 hours of Tumor Necrosis Factor-alpha (TNF-α) stimulation compared with control cells (Supporting Information Fig. S11F). Furthermore, the NFκB reporter assay confirmed the activation effect of Rai on the NFκB pathway. Rai overexpression in 293T cells significantly increased NFκB-dependent luciferase activity in basal conditions (Supporting Information Fig. S11G). Among the proinvasive genes showed in Figure 6A, we also found reduced expression levels of *ADAMs* in Rai-silenced NSs. ADAMs have been reported to cleave Notch, facilitating NICD release by γ-secretase [38]. We therefore analyzed the activation status of the Notch pathway in these cells. Interestingly, we observed a reduced expression of the intracellular active fragment of Notch1 (Fig. 7F) and a concomitant diminished expression of Notch target genes (data not shown). Among these, the transcription factor *SNAI* (Fig. 7G) was reported to be critical for tumor invasiveness in different cell types, including GBM cells [39–41]. Reactivation of Notch signaling in Rai-silenced NSs (Fig. 7H, 7I) fully reverted the migration defect (Fig. 7J). These data indicate that Rai induces migration/invasion through the activation of different patient-specific signaling pathways.

The Expression of Rai Is Higher in Proneural Subtype Tumors

Recently, gene expression studies have subclassified GBMs in molecular classes [32, 42]. Since we showed that Rai is involved in the activation of multiple pathways, it would be worthwhile to investigate whether Rai might be differentially expressed in the different molecular subclasses of GBMs.

We have thus performed a meta-analysis of Rai expression using the microarray datasets derived from Phillips [32] and the three independent microarray datasets comprising more than 200 GBMs derived from Verhaak [42]. We found that Rai was always significantly enriched in the proneural molecular subtype (Supporting Information Fig. S12A, S12B) known to be more frequently associated with Notch signaling activation [32]. Indeed, we found that Rai is highly expressed in the tumors which possess a higher expression of Notch (Supporting Information Fig. S12C). We then classified our 21 GBMs according to the three subtypes described by Phillips et al. using the signature of 35 genes published by the authors, and we established the subtype for 14 tumors (Supporting Information Table S4). Five out of 14 GBMs revealed to belong to the proneural subtype and, even if the number is low, they were among those expressing the higher levels of Rai. The GBM culture lines that we have used to silence Rai expression were derived from different GBM classes; notably, regardless of the GBM molecular subtype, Rai silencing always reduced tumor invasion.

Discussion

In this manuscript we demonstrate that Rai is a new regulator of cell migration and invasion, and that this is relevant both for the physiological migration of neural stem/progenitor cells and for the infiltration of GBM cancer stem/progenitor cells into normal parenchyma. Normal and cancer stem/progenitor cells share the ability to migrate throughout the brain. Neural stem cells residing in the SVZ generate neuroblasts, which migrate a long distance to reach their final destination in the OB, where they differentiate into neurons. Cancer stem cells possess high infiltrative capability: a high proportion of cells expressing stem cell markers has been found at the tumor/host interface in brain tumors [43, 44] and in metastasis of other solid tumors [45–47]. Indeed, it has been proposed to exploit the high invasiveness of GBM stem cells as a new method to enrich the cancer stem cell population [48]; recently, a greater invasive potential of GBM stem cells compared with matched nonstem tumor cells has been reported both in vitro and in vivo [49]. Rai is expressed in neural stem/progenitor cells and its expression is maintained physiologically in neurons but not glial cells. The physiological function of Rai in neural progenitors is also retained in cancer stem/progenitor cells conferring them a migratory and invasive phenotype. We show that the few Rai-positive tumor cells in Rai-interfered xenografts are localized at the tumor-host interface and express the immature cell marker Olig2 and the promigratory Dcx protein. Under physiological conditions, Dcx regulates migration of embryonic and adult SVZ neural progenitors [50, 51]. Dcx expression has been found in invasive human gliomas with highest intensity at the invasive

Figure 7. Rai acts through multiple pathways to regulate cell migration and infiltration. **(A):** Western blotting: increased phosphorylation level of β-catenin in Rai−/− NSs compared with WT. The antibody used recognizes two phosphorylated residues of β-catenin, Threonine 41, phosphorylated by GSK-3β, and Serine 45, phosphorylated by Casein Kinase I (CKI). **(B):** Confocal immunofluorescence analysis of WT and Rai−/− NSs left untreated (left panel), leptomycin B treated (middle panel) or leptomycin B + Wnt-3a treated (right panel), using an antibody against β-catenin (red) to reveal its nuclear translocation; nuclei were stained with DAPI (blue). In standard culture conditions, no β-catenin staining is visible in WT or Rai−/− nuclei (left panel). Since this might be due to active nuclear export of β-catenin, cells were treated with the nuclear export inhibitor leptomycin B: a small amount of β-catenin in the nuclei of WT but not Rai−/− NSs is detectable (middle panel). Leptomycin B-treated NSs were then stimulated with Wnt-3a for 2 hours: β-catenin nuclear translocation is clearly detectable in WT but not in Rai−/− cells (right panel). **(C):** Quantitative real time polymerase chain reaction (qRT-PCR): downregulation of neurogenesis-specific β-catenin target genes in Rai−/− NSs; mean ± SD, **, $p < .01$. **(D):** Western blotting: reduction in the activating phosphorylation of the Serine-residue 536 of the NFκB p65 subunit in Rai-silenced hGBM#7 NSs after TNF-α stimulation for the indicated time points. **(E):** Boyden chamber assay: migration is restored in Rai-silenced hGBM#7 NSs transduced with a lentiviral vector carrying *p65* cDNA. **(F):** Western blotting: reduced activity of the Notch pathway in Rai-silenced GBM NSs, using an antibody specific for NICD. **(G):** qRT-PCR: reduced expression of the Notch target gene *SNAI2* in Rai-silenced GBM NSs; mean ± SD, **, $p < .01$. **(H):** Western blotting: reactivation of the Notch pathway in Rai-silenced GBM NSs transduced with a lentiviral vector carrying *NICD* cDNA. **(I):** qRT-PCR: increased expression of the Notch target gene *SNAI2* in Rai-silenced GBM NSs infected with a lentiviral vector carrying *NICD* cDNA; mean ± SD, **, $p < .01$. **(J):** Boyden chamber assay: migration is restored in Rai-silenced GBM NSs transduced with a lentiviral vector carrying *NICD* cDNA (Supporting Information Figs. S11, S12). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; *EBF3, Early B-cell Factor 3*; hGBM, human glioblastoma; *NEUROD1, Neurogenic Differentiation 1*; *NGN2, neurogenin2*; *NICD*, *notch intracellular domain*; NSs, neurospheres; NT shRNA, nontargeting short hairpin RNA; TNF-α, Tumor Necrosis Factor-alpha; WT, wild type.

front [23], and it has been proposed as a specific marker for the identification of individual infiltrating glioma cells [24]. We found a significant reduction in the number of invading cells positive for Dcx in all Rai-interfered xenografts examined as well as a decreased amount of Dcx in both Rai−/− stem/progenitor cells and in Rai-interfered tumor stem/progenitor cells. This striking correlation between the expression levels of Rai and Dcx strongly suggests that Rai may regulate Dcx expression. However, the regulation of Dcx expression is still poorly understood [52, 53]. Interestingly, Ngn2, a β-catenin target [34], directly binds to the Dcx promoter both in cultured embryonic neural progenitors and in vivo in the developing mouse cortex, promoting cell migration [54]; recent studies have identified an upstream promoter region of Dcx containing binding sites for lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors [55], which are central effectors of the Wnt/β-catenin pathway. The impaired Wnt/β-catenin signaling and the consequent *NGN2* downregulation that we have observed in the "nontumoral" Rai−/− stem/progenitor cells might help to explain the reduced Dcx expression in these cells. On the other hand, we did not find any alteration of Wnt signaling in any of the Rai-silenced GBM NSs tested. In the absence of Rai, we always found reduced expression of different types of hydrolytic enzymes, including *MMPs* and *ADAMs* in both normal and tumor stem/progenitor cells; this finding could be the consequence of the alteration of different signaling pathways, such as PI3K/Akt, NFκB, and Notch. In contrast with what happens in mature neurons, Rai does not regulate PI3K/Akt signaling neither in normal neural progenitors nor in GBM. This context-dependent function of Rai has been already described in lymphocytes, where Rai negatively regulates Akt signaling [56]. Interestingly, recent data showed that Akt isoforms induce different biological phenotypes in astrocytes and in glioma cells with different genetic mutations [57]. Thus, it appears plausible that Rai functioning as an upstream activator of the PI3K/Akt pathway could be displaced in glioma cells in the presence of frequent genetic alterations like *PTEN* mutations or *EGFR* amplification playing a similar role.

The promoter region of *MMP9* contains putative binding sites for NFkB [37] and the blockade of the transcriptional activity of NFκB in human GBM cells contributes to the inhibition of cell invasion [58, 59]. However, despite similar in vivo and in vitro phenotypes, we found disruption of the NFκB pathway only in Rai-silenced NSs derived from one out of eight GBM patients, while in other Rai-silenced GBM-derived NSs we found that Notch signaling was downregulated, reflecting the genetic heterogeneity of this kind of tumor. Notch signaling is implicated in the maintenance of neural progenitors [60] and in glioma initiation and tumor formation [61]. Little is known about its involvement in glioma cell migration [62, 63]. Interestingly, in different tumor cells, including glioma cells, hypoxia-induced increased cell motility is mediated by activation of Notch signaling, which controls the expression of the Snai zinc finger transcription factor family [39, 40]. Here, we show that the reduction in Notch signaling observed in Rai-targeted NSs is accompanied by diminished expression of the transcription factor *SNAI2* that could explain both reduced invasion and migration. Rai ability to activate multiple-signaling pathways is not surprising considering that it is an adaptor protein and so it is intrinsic in its function.

Multiple genetic alterations and different signaling pathways have been identified as responsible for GBM initiation, migration, and invasion, and combination therapies directed at multiple targets are becoming common in clinical trials. The complexity of this type of tumor is increased by the discovery of differences in the phenotype of cancer stem cells derived from tumors of equivalent histological class and grade [64, 65]. Future treatments will have to take into account this heterogeneity. Thus, finding a target common to all GBM cancer stem cells derived from different patients might be ground-breaking. This common target might be Rai, since targeting Rai in GBMs would reduce migration and invasion independent of the different signaling pathways that may be activated in the various patients. However, the prosurvival and the promigratory effect that Rai exerts in physiological conditions on mature neurons and neural progenitors, respectively, raise concerns on the opportunity of using Rai silencing for therapeutic use in GBM treatment. Determining the molecular mechanisms responsible for the different state of Rai activation in tumoral versus normal stem/progenitor cells could allow specific targeting of cancer cells. We have previously described a likely mechanism of activation of the Shc proteins in different tumors that involves their constitutive tyrosine phosphorylation [66]. However, Rai was not constitutively tyrosine phosphorylated in GBM NSs (our unpublished results) suggesting that tyrosine phosphorylation is not the mechanism responsible for Rai-dependent cell migration. We are currently testing the possibility that Rai serine-threonine phosphorylation, described in mature neurons after stress stimuli, could be operating also in glioma cells.

Recently, GBMs have been classified by gene expression signatures in four distinct subtypes (from The Cancer Genome Atlas Research Network: proneural, neural, classic, and mesenchymal) [13], which are not dissimilar from the three subtypes (proneural, proliferative, and mesenchymal) described by Phillips et al., and are characterized by the prevalent activation of specific signaling pathways. We investigated whether *Rai* was differentially expressed in the different molecular subclasses of GBMs and found that Rai was overexpressed in the proneural type, and the pattern of Rai distribution was variable within the different subtypes. The proneural subtype expresses genes associated with the neurogenic process and which are markers of neuroblasts or developing neurons, such as Dcx. Thus, it is not surprising to find the highest levels of Rai expression in the GBM subtype, which shows a prevalence of developing neurons or neuroblasts, particularly in view of the restricted pattern of Rai expression in normal mature neurons and neuroblasts and of the persistence of its expression in GBM tumor-initiating cells. Interestingly, the GBM cell culture lines that we have used to silence *Rai* expression were derived from different GBM subclasses, indicating that Rai silencing always reduced tumor invasion, independently of the GBM molecular subtype from which the NSs were isolated.

Conclusions

Rai is a new regulator of normal and cancer stem/progenitor cell migration. Targeting Rai in GBMs reduces migration and invasion, regardless of the different signaling pathways that may be activated in the various patients. These findings support the potential use of Rai as a new target to block brain cancer invasion.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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