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Role of Rac1-regulated signaling in medulloblastoma invasion

Laboratory investigation

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Object. Medulloblastomas are the most common malignant brain tumors in children. These tumors are highly invasive, and patients harboring these lesions are frequently diagnosed with distant spread. In this study, the authors investigated the role of Rac1, a member of the Rho family of small guanosine triphosphatases, in medulloblastoma invasion.

Methods. Three established medulloblastoma cell lines were used: DAOY, UW-228, and ONS-76. Specific depletion of Rac1 protein was accomplished by transient transfection of small interfering RNA. Cell invasion through extracellular matrix (Matrigel) was quantified using a transwell migration assay. Mitogen activated protein kinase activation was determined using phospho-MAP kinase–specific antibodies, and inhibition of MAP kinase pathways was achieved by specific small molecule inhibitors. Localization of Rac1 and its expression levels were determined by immunohistochemical analysis using a Rac1-specific antibody, and Rac1 activation was qualitatively assessed by Rac1 plasma membrane association.

Results. Small interfering RNA–mediated depletion of Rac1 strongly inhibited medulloblastoma cell invasion. Although depletion of Rac1 inhibited the proliferation of UW-228 cells, and of ONS-76 cells to a lesser extent, it stimulated the proliferation of DAOY cells. Depletion of Rac1 also inhibited the activation of the ERK and JNK MAP kinase pathways, and inhibition of either pathway diminished invasion and proliferation. Immunohistochemical analysis demonstrated that the Rac1 protein was overexpressed in all medulloblastoma tumors examined, and indicated that Rac1 was hyperactive in 6 of 25 tumors.

Conclusions. The authors' data show that Rac1 is necessary for the invasive behavior of medulloblastoma cells in vitro, and plays a variable role in medulloblastoma cell proliferation. In addition, these results indicate that Rac1 stimulates medulloblastoma invasion by activating the ERK and JNK pathways. The authors suggest that Rac1 and signaling elements controlled by this guanosine triphosphatase may serve as novel targets for therapeutic intervention in malignant medulloblastomas. *(DOI: 10.3171/2009.4.PEDS08322)*

Key Words • medulloblastoma • invasion • Rac1 • ERK • JNK

Medulloblastomas are highly cellular, primitive neuroectodermal tumors of the posterior fossa. They are the most common malignant brain tumor in children, representing up to 25% of all childhood brain tumors, and 40% of all childhood posterior fossa tumors.[14,28] The prognosis is largely dependent on age, extent of disease spread (based on the presence of tumor cells in the CSF and on pre- and postoperative neuroimaging), and the extent of resection (< 1.5 cm^3 of residual tumor).[8,45] In children who undergo adequate resection followed by aggressive adjunctive treatment consisting of radio- and chemotherapy, 5-year overall survival rates as high as 85% have been reported in those with focal tumors, and as high as 70% in patients with invasive disease.[18] Of patients who do survive, however, many exhibit long-term neurocognitive and/or neuroendocrine sequelae. Metastatic spread is present in 11–43% of patients with medulloblastomas,[8] yet to date very little is understood concerning the genet-

Abbreviations used in this paper: FBS = fetal bovine serum; GEF = guanine nucleotide exchange factor; GTP = guanosine triphosphate; MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; siRNA = small interfering RNA; SRB = sulphorhodamine B.

ics and biological mechanisms altered that allow invasion and distant spread in patients with high-risk medulloblastomas.

Recent genomic analysis has revealed the existence of 5 subtypes that have distinct genetic profiles and pathological features and appear to be driven by different signaling pathways.[25,38] Notably, tumors characterized by increased protein synthesis, cell cycle activity, and photoreceptor differentiation have a strong tendency to display metastatic disease.[25] The mechanistic implications of these findings still need to be determined, however.

Work from our laboratory and several others has shown that the small GTPase Rac1 plays a critical role in the invasive behavior of several tumor types.[4,9,13,34] As do other members of the Rho family of Ras-like small GTPases, Rac1 essentially functions as a switch: it is "on" in the GTP-bound state, and "off" in the guanosine diphosphate–bound state.[37] In the active state, Rac1 relays signals from growth factors, cytokines, and adhesion molecules to a large number of effector proteins. These effectors in turn initiate distinct signaling cascades that work in concert to regulate the organization of the actin cytoskeleton, and cell adhesion, migration, invasion, and proliferation.[24,33]

In this study, we use RNA interference directed against Rac1 and small molecule inhibitors of MAP kinase pathways to investigate the role of Rac1 and Rac1-controlled signaling elements in the invasive behavior of medulloblastoma. We show that Rac1 is essential for medulloblastoma cell invasion and provide evidence that the ERK and JNK pathways function downstream of Rac1 to stimulate medulloblastoma invasion.

Methods

Reagents Used

The following antibodies were used for Western blot analysis: anti-Rac1 (Upstate Biotechnology), anti–phospho-ERK1/2 (Thr202/Tyr204), anti–phospho-JNK 1/2 (Thr183/Tyr 185), anti–phospho-p38 (Thr 180/Tyr 182), anti–phospho-p70S6 (Ser 389), and anti-tubulin, all purchased from Cell Signaling Technology. Small molecule inhibitors were purchased from Promega (U0126) and Calbiochem (SP600125 and SB203580). Sulphorhodamine B was purchased from Sigma.

Cell Lines and Tissue Culture Conditions

The human medulloblastoma cell line DAOY was obtained from American Type Culture Collection, and the UW-228 and ONS-76 medulloblastoma cell lines were kindly provided by Dr. James Rutka (The Hospital for Sick Children, University of Toronto Hospital). All 3 cell lines grow as adherent monolayers and were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified 5% CO_2 incubator.

Small Interfering RNA and Transfection Conditions

All siRNA target sequences were purchased from Dharmacon in deprotected and desalted form, and siRNA oligonucleotide duplexes specific for Rac1 were designed as previously described.[4,12] We used the following siRNA sequences: Rac1–1, corresponding to bp 439–459 after the start codon of the *Rac1* gene (5′AAGGAGATTGGTG CTGTAAAA); Rac1–2, corresponding to bp 616–636 after the start codon of the *Rac1* gene (5′AACCTTTGTA CGCTTTGCTCA); and luciferase, corresponding to bp 291–309 after the start codon of the *GL2* luciferase gene (5′AACGTACGCGGAATACTTCGA). For siRNA transfection, cells were plated at a density of 10^5 cells per well in a 6-well plate for 4 hours, and then transfected using 20 nM of the siRNA duplex with 2.2 μg/ml of lipofectamine 2000 (Invitrogen). Cells were transfected for 24 hours, and then fresh DMEM supplemented with 10% FBS and 1% penicillin-streptomycin was added.

Western Blot Analysis

Three days posttransfection, cells were starved overnight in DMEM and 0.5% bovine serum albumin, and subsequently stimulated with DMEM and 10% FBS for 5 minutes. Cells were rinsed with cold phosphate-buffered saline and lysed in radioimmunoprecipitation assay buffer. Fifteen micrograms of proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose polyvinylidene fluoride membranes for Western blot analysis. Goat primary phospho-specific antibodies were diluted 1:1000 in 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 (TBST) and mouse primary Rac1-specific antibodies were diluted 1:2000 in 5% milk in TBST. Blots were developed utilizing SuperSignal West Pico enhanced chemiluminescence detection (Pierce), and scanned and quantified using Image J software (National Institutes of Health).

Invasion Assays

Invasion assays were performed essentially as previously described.[39] Briefly, Matrigel invasion chambers (BD Falcon or Chemicon/Millipore) were hydrated in serum-free DMEM for 2 hours and then placed in DMEM with 10% FBS and 1% penicillin-streptomycin (bottom compartment). Three days after siRNA transfection, the cells were plated in the top well of prehydrated Matrigel invasion chambers (BD Falcon or Chemicon/Millipore) at a density of 2×10^4 cells. Both wells were provided with DMEM and 10% FBS. Cells were allowed to invade for 24 hours. Filters were fixed in 3.7% formaldehyde (Sigma) in phosphate-buffered saline for 15 minutes and subsequently stained with 0.2% crystal violet in 2% ethanol for 10 minutes. Invasion was quantified by counting of the total cell number on the underside of the filter using an objective lens with a magnification of 10 on an IX70 Olympus inverted microscope equipped with a digital camera.

Immunohistochemical Analysis

Sections (4-μm thick) were quenched with 3% hydrogen peroxide in methanol for 20 minutes, treated in a microwave oven for 3 minutes at 500 W using a citrate buffer (pH 6.0). After blocking nonspecific binding with 10% horse serum, they were incubated with mouse monoclonal antibodies against Rac1 (1:200) or nonimmune mouse IgG (30 μg/ml) overnight at 4°C. After reactions with biotinylated horse IgG to mouse IgG and an avidin-biotin-peroxidase complex (Vector Laboratories), the color was developed with 3,3'-di-

aminobenzidine tetrahydrochloride (Vector Laboratories). Counterstaining was performed with hematoxylin.

Sulphorhodamine B and MTS Assays

Cell proliferation was measured using the SRB colorimetric assay.[36] Briefly, 1 day after siRNA transfection, cells were seeded at 2 × 10^3 cells/well in 6 wells of a 96-well plate. At various times, cells were fixed in 10% trichloroacetic acid for 1 hour at 4°C, rinsed, and subsequently stained for 30 minutes at room temperature with 0.2% SRB dissolved in 1% acetic acid, followed by air drying. The bound dye was solubilized in 100 µl of 10-mM unbuffered Tris base for 30 minutes, and absorbance was read at 490 nm in an enzyme-linked immunosorbent plate reader. The MTS assay was performed on Day 3 after transfection with siRNA, targeting either Rac1 or luciferase using CellTiter 96 (Promega) following the manufacturer's protocol.

Results

To study the role of Rac1 in medulloblastoma invasion, we depleted Rac1 using transient transfection of siRNA[11,12] and determined the effect of Rac1 depletion on cell invasion through a thin layer of reconstituted extracellular matrix (Matrigel), a standard assay for quantifying the invasive behavior of a large variety of cancer cells.[39] We also note that the major components of Matrigel are laminin and collagen IV, both of which are enriched in the leptomeningeal extracellular matrix.[41] Depletion of Rac1 strongly inhibits the invasive behavior of all 3 medulloblastoma cell lines we examined, DAOY, UW-228, and ONS-76 (Fig. 1). We obtained similar inhibition of cell invasion with 2 independent siRNA oligonucleotides that we previously characterized,[4] confirming the specificity of these reagents.

We used the colorimetric MTS assay to examine whether depletion of Rac1 has an effect on the survival of medulloblastoma cells, thereby indirectly affecting their invasive behavior. Three days after siRNA transfection, the time when optimal depletion is achieved, the MTS assay values for Rac1-depleted medulloblastoma cells were virtually identical to those of control cells: 99 ± 3% for Rac1-depleted DAOY cells compared to control transfected DAOY cells, and 101 ± 2% for Rac1-depleted UW-228 cells compared to control transfected UW-228 cells. Thus, depletion of Rac1 did not have any effect on medulloblastoma cell survival. We also examined the effect of Rac1 depletion on cell proliferation using the colorimetric SRB assay.[36] Surprisingly, the role of Rac1 in medulloblastoma cell proliferation qualitatively depends on the cell line studied. Although depletion of Rac1 inhibits the proliferation of UW-228 cells and to a lesser extent that of ONS-76 cells, it has a stimulatory effect on the proliferation of DAOY cells (Fig. 2). No significant proliferation was observed, however, over the first day of plating, which corresponds to the time period during which the invasion assay took place; this rules out the possibility that the role of Rac1 in the proliferative behavior of the respective cell lines significantly modulates the observed effect of Rac1 depletion on the extent of invasion of these cells.

The role of Rac1 in medulloblastoma invasion raised the question of whether there is an increase in Rac1 protein expression in medulloblastoma tissues. We therefore performed immunohistochemical analysis on a panel of 25 medulloblastoma cases, including specimens from 6 patients in whom CNS dissemination was displayed at diagnosis. All tumor samples stained positively for Rac1, while normal cerebellum showed no significant staining (Fig. 3). Notably, whereas Rac1 staining was predominantly cytoplasmic in the majority of the cases, in 6 specimens, there was marked plasma membrane staining in addition to cytosolic staining of Rac1. This finding is significant in that inactive Rac1 resides in the cytoplasm, where it is bound to guanosine diphosphate dissociation inhibitors, whereas activated Rac1 localizes to the plasma membrane.[29] Thus, these observations indicate a high level of Rac1 activation in a subset of medulloblastoma samples. No correlation be-

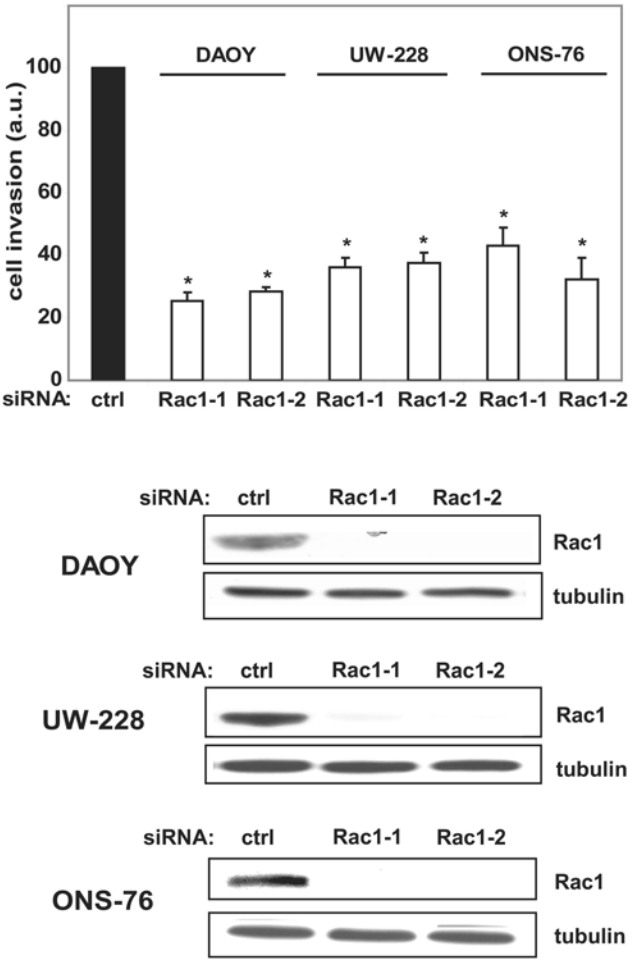

Fig. 1. The presence of Rac1 is necessary for medulloblastoma cell invasion. *Upper:* Graph showing Matrigel invasion assay. The DAOY, UW-228, and ONS-76 medulloblastoma cells were transfected with siRNAs targeting 2 different regions of the *Rac1* gene (Rac1–1 and Rac1–2) or luciferase (control [ctrl]). Three days after transfection, cells were seeded onto Matrigel-coated filters and allowed to invade for 24 hours against a gradient of 10% FBS. Subsequently, cells were fixed, stained, and the total number of invaded cells was determined using digital microscopy. Invaded cells were normalized to invaded control cells. Data shown are the means of 3–6 independent experiments ± SEMs, using triplicate wells each. *p < 0.001, 2-tailed t-test, compared to control. *Lower:* Western blot analysis verifying depletion of Rac1 in the 3 cell lines. a.u. = arbitrary units.

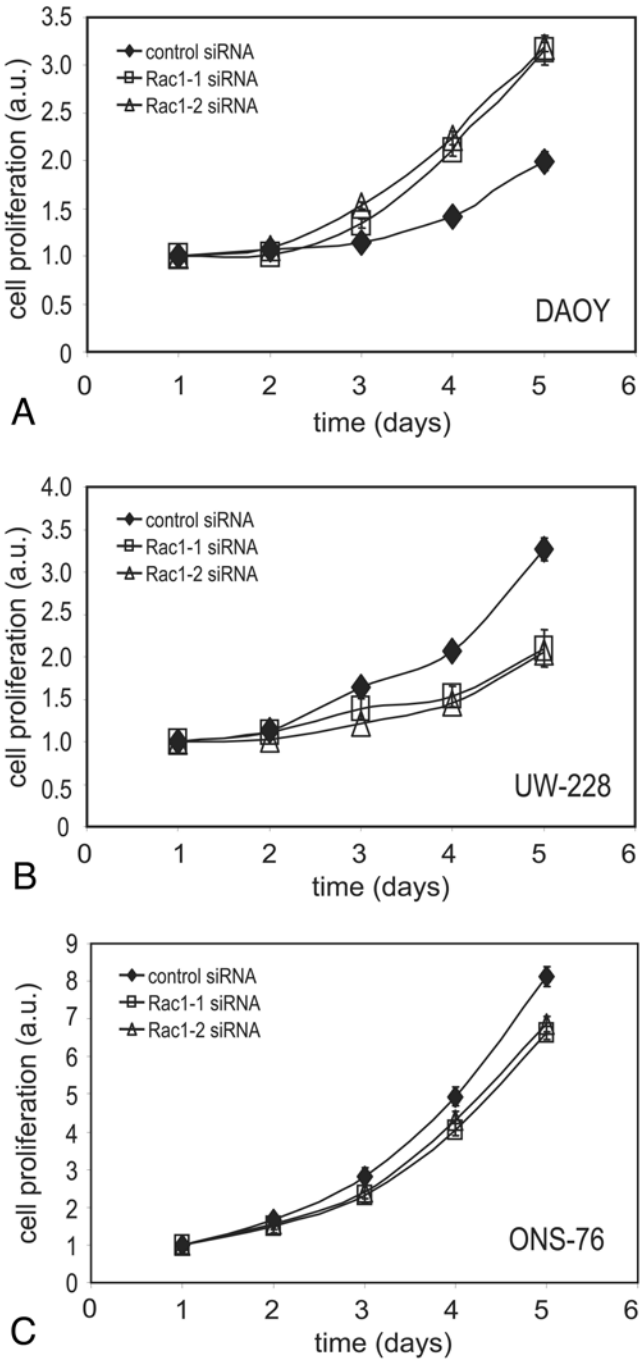

Fig. 2. Graphs showing the role of Rac1 in the proliferative behavior of medulloblastoma cells. The DAOY (A), UW-228 (B), and ONS-76 cells (C) were transfected with siRNAs targeting either Rac1 or luciferase (control). One day after transfection, cells were seeded in 96-well plates in serum-containing medium. Cells were fixed 8 hours after seeding (Day 1) to allow cells to adhere, and subsequently at 24-hour intervals. The SRB staining and readings were performed as described in *Methods*. Values for Days 2–5 were normalized to those obtained on Day 1 after transfection. Data shown are the means of 6 wells ± SEMs and are representative of 5 independent experiments.

tween Rac1 plasma membrane staining and CNS dissemination was observed, however.

To begin elucidating the signal transduction mechanisms that underlie the role of Rac1 in medulloblastoma invasion, we first examined the roles of the 3 MAP kinase signaling cascades (ERK, JNK, and p38) in this process, because Rac1 has been shown to contribute to their activation.[7,17,26,27] To inhibit the respective MAP kinases, we used chemical inhibitors that specifically interfered either with the kinases themselves or with upstream elements. Inhibition of either MEK1/2, the kinases that phosphorylate and activate ERK by U0126[15] or of JNK by SP600125[22] significantly inhibited the invasive behavior of both DAOY and UW-228 cells (Fig. 4). Inhibition of p38α/β by SB203580[44] at a concentration that strongly inhibits these kinases[6] had no significant effect on the invasiveness of the medulloblastoma cells (Fig. 4). These results indicate that both the ERK and JNK kinases are necessary for medulloblastoma invasion.

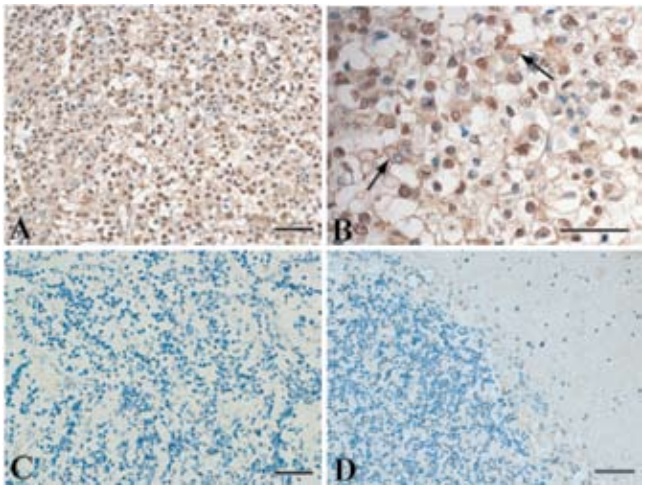

Fig. 3. Photomicrographs showing the immunolocalization of Rac1 in medulloblastoma cells. Paraffin sections were immunostained with monoclonal antibodies against Rac1 (A and B). Note the Rac1 immunostaining on the cell membrane of medulloblastoma cells *(arrows)*. No significant staining is observed in medulloblastoma sections stained with nonimmune IgG (C) and normal cerebellum (granular layer *[left]* and molecular layer *[right]*) with anti-Rac1 antibody (D). Hematoxylin counterstain. Bar = 100 μm.

We also examined the role of these signaling pathways in medulloblastoma cell proliferation. As is the case for cell invasion, inhibition of either the ERK or JNK pathways significantly inhibited the invasive behavior of both DAOY and UW-228 cells, whereas inhibition of p38 had only a minor inhibitory effect (Fig. 5).

We next investigated the extent to which Rac1 contributes to the activity of the ERK, JNK, and p38 kinases in medulloblastoma cells. To accomplish this, we determined whether siRNA-mediated depletion of Rac1 inhibits the activation of these MAP kinases by serum stimulation, using Western blot analysis with antibodies specific for the activated state of these kinases. We observed that depletion of Rac1 significantly inhibits serum-stimulated activation of ERK and JNK in both DAOY and UW-228 cells (Figs. 6 and 7). Surprisingly, however, inhibition of Rac1 expression enhanced serum-stimulated activation of p38 in the medulloblastoma cells (Fig. 8). Thus, taken together, our observations indicate that Rac1 regulates medulloblastoma invasion by stimulating the ERK and JNK pathways.

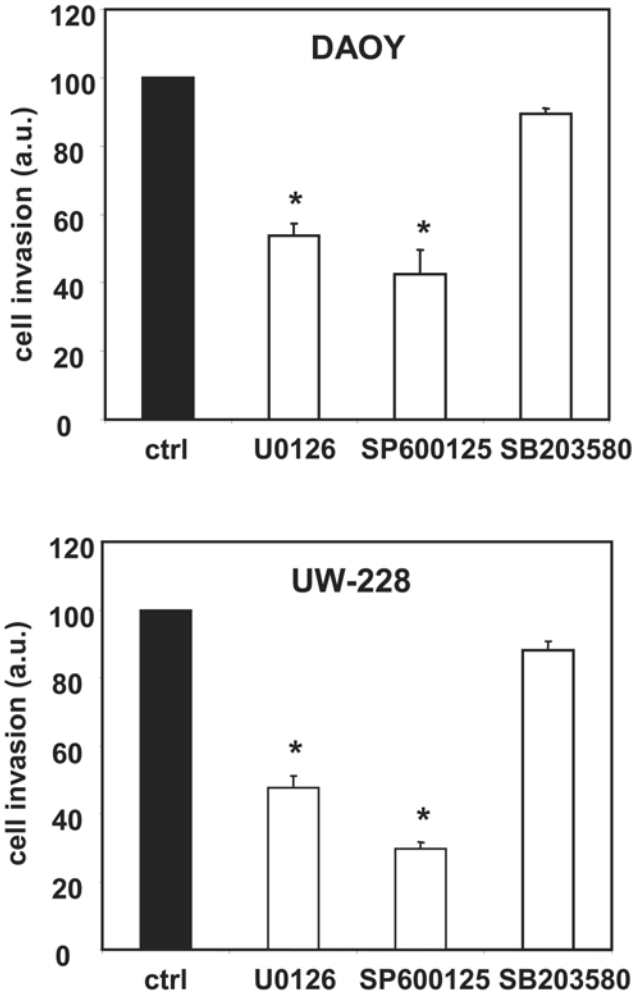

FIG. 4. Graphs showing that activation of the ERK and JNK pathways is necessary for medulloblastoma cell invasion in DAOY cells *(upper)* and UW-228 cells *(lower)*. Invasion assays were performed as described in Fig. 1. Small molecule inhibitors of MEK (U0126, 2 μM), JNK (SP600125, 20 μM), or p38 (SB203580, 2 μM) were added to both top and bottom wells. Control cells were treated with the equivalent volume of DMSO. Data shown are the means ± SEMs of 4 experiments using triplicate wells each. *$p < 0.001$, 2-tailed t-test.

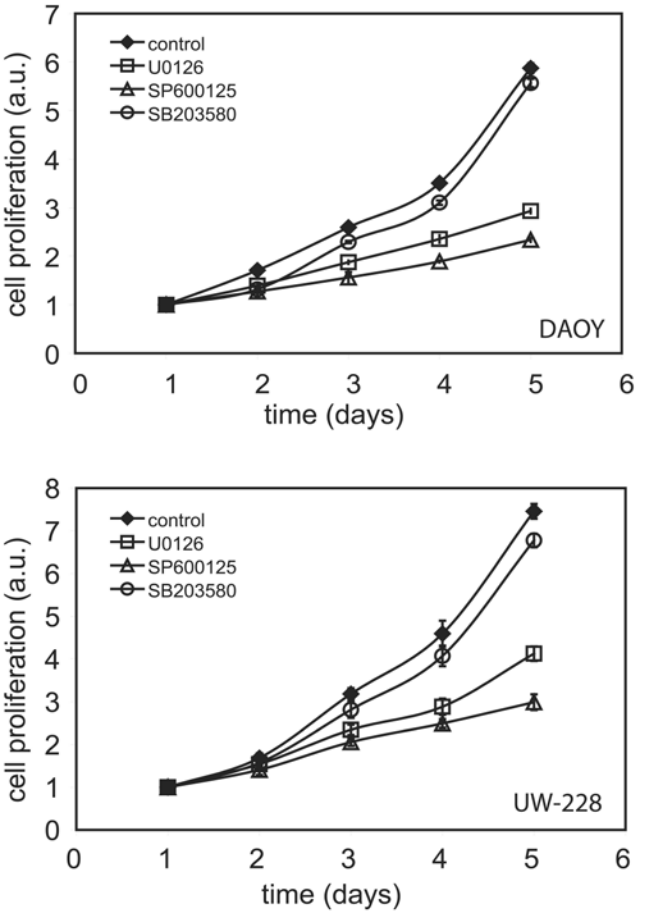

FIG. 5. Graphs showing that activation of the ERK and JNK pathways is necessary for medulloblastoma cell proliferation in DAOY cells *(upper)* and UW-228 cells *(lower)*. Cells were plated as described in Fig. 2 in the presence of U0126 (2 μM), SP600125 (20 μM), or SB203580 (2 μM). Control cells were treated with the equivalent volume of DMSO; inhibitors were replenished every other day. Data shown are the means of 6 wells ± SEMs and are representative of 3 independent experiments.

Discussion

Our data in the present study demonstrate an essential role for the small GTPase Rac1 in the invasive behavior of medulloblastoma cells. Our data further suggest that the ERK and JNK pathways act downstream of Rac1 to stimulate medulloblastoma invasion. Importantly, immunohistochemical analysis also demonstrated that Rac1 is overexpressed and hyperactive in medulloblastoma tissue in situ.

Overexpression of Rac1 protein has been observed in a large number of different tumors.[20] However, knowledge about the activation state of Rac1 in tumors is still lacking, although this information is highly relevant to clinical treatment in patients with cancer. Thus, our data suggesting that Rac1 is hyperactive in a subset of medulloblastoma cases is very significant; we recently made similar observations in glioblastomas.[35]

Medulloblastoma cells display deregulation of a number of tyrosine kinase receptors, and in some instances of their cognate ligands, which could lead to constitutive activation of Rac1, including c-Met, ErbB2, IGF receptor, and PDGF receptor β.[19,21] All these tyrosine kinase receptors have been shown to cause Rac1 activation in other cell systems[2,3,43] and therefore probably also in medulloblastoma cells.

Our data also show, for the first time, that both the ERK and JNK pathways contribute to the invasive behavior of medulloblastoma cells. Notably, the ERK pathway is upregulated in a large fraction of medulloblastoma tumors.[42] It will therefore also be of interest to investigate whether JNK is upregulated in medulloblastoma cells.

The role of the ERK pathway in tumor cell invasion is well documented, and is mediated by directly modulating the function of cytosolic proteins such as MLCK, and stimulating the expression of many genes, including matrix metalloproteases.[31] The JNK pathway has also been implicated in the control of cell migration, and similar to ERK, phosphorylates cytosolic substrates such as the focal adhesion protein paxillin and stimulates the transcription of metalloproteases.[23]

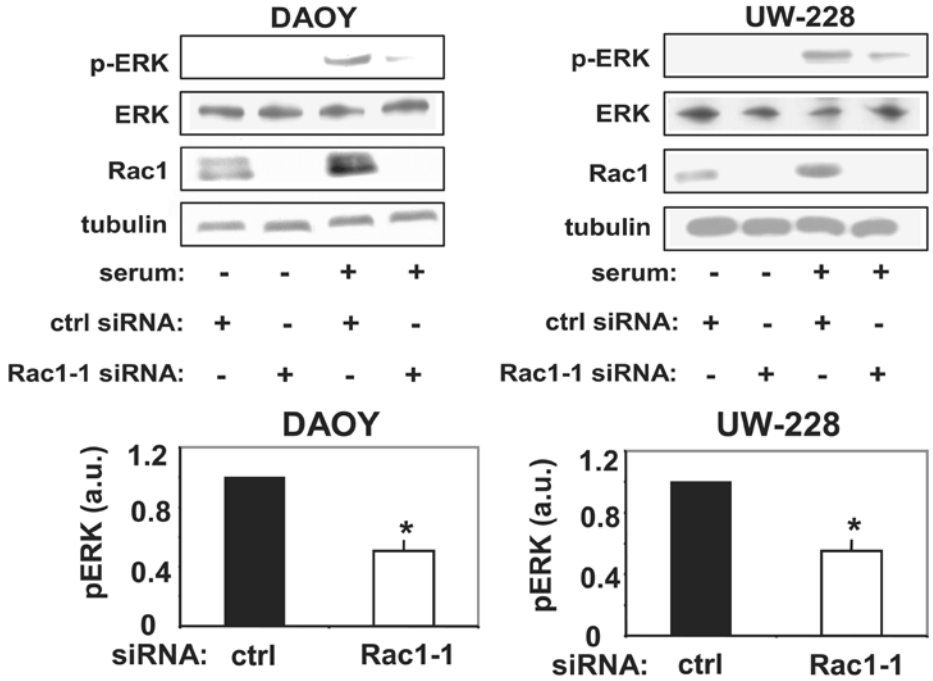

FIG. 6. The presence of Rac1 is necessary for efficient ERK activation in medulloblastoma cells. *Upper:* Western blots showing serum-induced ERK activation (phospho-ERK [p-ERK]) in Rac1-depleted and control cells. *Lower:* Bar graphs showing quantification of the Western blots. Data shown are the means ± SEMs of 3 experiments. *p < 0.001, 2-tailed t-test.

It is likely, however, that in addition to mediating the activation of the ERK and JNK pathways, Rac1 stimulates additional signaling pathways to promote cell invasion. In line with this, we found that the phosphatidylinositol phosphatase synaptojanin 2, an effector of Rac1 that is essential for tumor cell invasion (including in glioblastoma cells[5]) is also necessary for invasion of medulloblastoma cells (data not shown).

Although Rac1 is critical to the invasive behavior of the 3 medulloblastoma cell lines tested, we found that it had a variable role in medulloblastoma cell proliferation, as the effects of Rac1 depletion on cell proliferation qualitatively depended on the cell line used. The stimulatory effect of depleting Rac1 in DAOY cells is surprising, as inhibition of Rac1 activity generally leads to a decrease in cell proliferation.[20,40] Interestingly, our analysis of Rac1-controlled

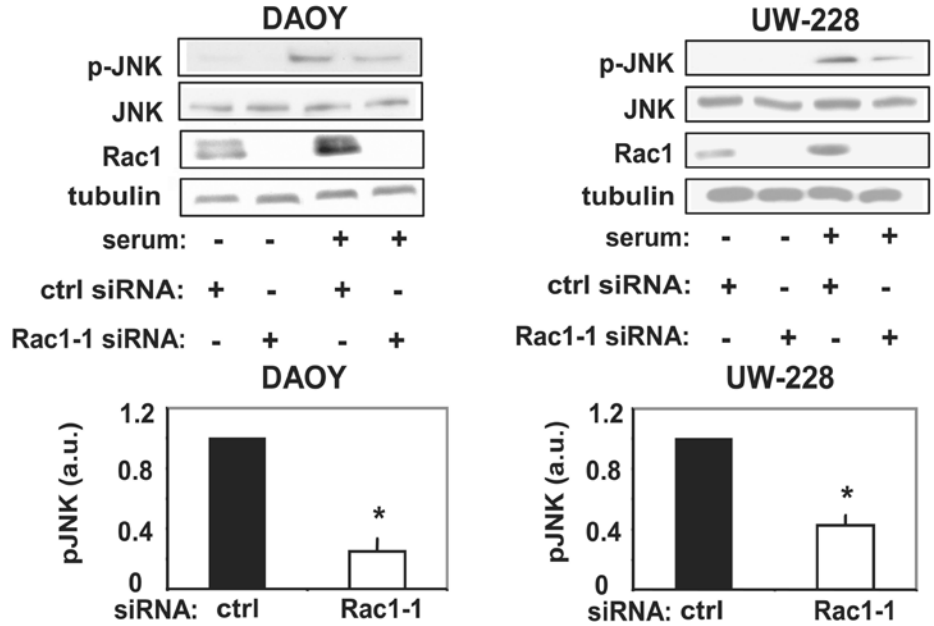

FIG. 7. The presence of Rac1 is necessary for efficient JNK activation in medulloblastoma cells. *Upper:* Western blots showing serum-induced JNK activation (phospho-JNK [p-JNK]) in Rac1-depleted and control cells. *Lower:* Bar graphs showing quantification of the Western blots. Data shown are the means ± SEMs of 3 experiments. *p < 0.001, 2-tailed t-test.

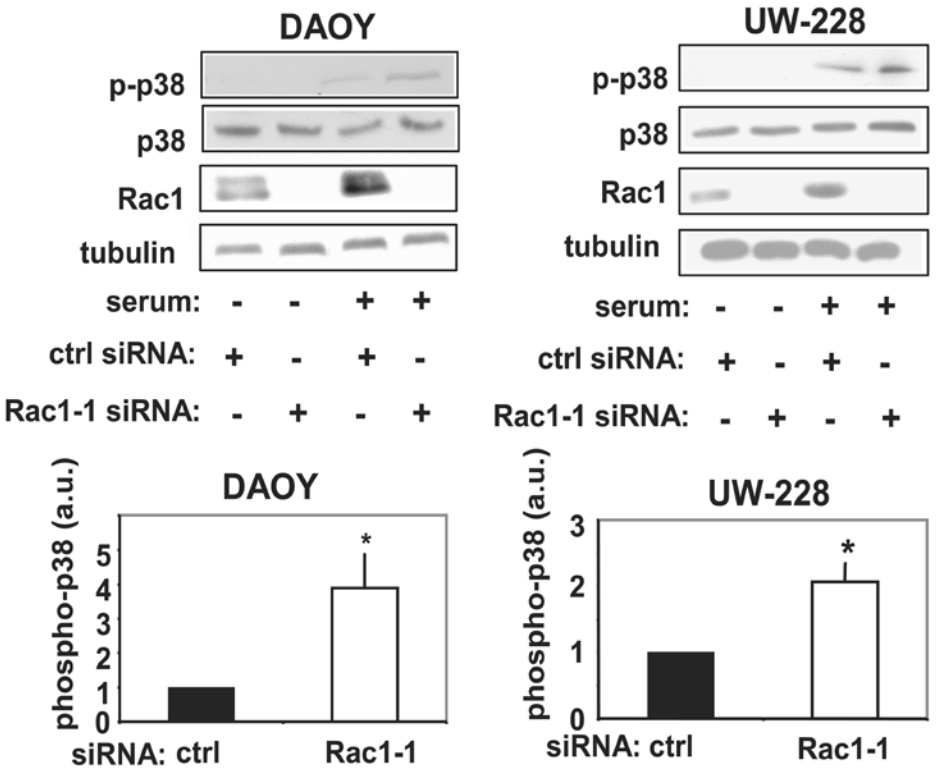

FIG. 8. Depletion of Rac1 enhances p38 activation in medulloblastoma cells. *Upper:* Western blots showing serum-induced p38 activation (phospho-p38 [p-p38]) in Rac1-depleted and control cells. *Lower:* Bar graphs showing quantification of Western blots. Data shown are the means ± SEMs of 3 experiments. *p < 0.001, 2-tailed t-test.

signaling pathways demonstrated that Rac1 is necessary for serum-stimulated activation of the ERK and JNK pathways, both in DAOY and UW-228 cells. Moreover, we found that interference with the ERK and JNK pathways strongly diminished cell proliferation in both DAOY and UW-228 cells, in accordance with the proproliferative roles of these pathways in other cells.[30] Therefore, the signaling mechanisms responsible for the inhibitory effect of Rac1 on DAOY cell proliferation are not mediated by either the ERK or JNK pathway, and remain to be elucidated.

Conclusions

Our results indicate the critical role of Rac1 in the invasive behavior of medulloblastoma cells. Whether Rac1 represents a novel potential therapeutic target for medulloblastoma remains to be critically evaluated.[16] An important caveat is that Rac1 controls a large number of cellular functions.[24] In particular, Rac1 plays a critical role in the migratory properties of a number of normal cell types, including neurons and leukocytes.[1,10] However, our findings strongly suggest that further molecular dissection of Rac1-controlled signaling mechanisms may yield additional drug targets for medulloblastoma therapy. For instance, there are a large number of GEFs that have been shown to activate Rac1[32] and are likely to offer a larger therapeutic window. Indeed, a number of these GEFs are expressed in a tissue- or cell type–specific manner, and/or may be hyperactive in medulloblastoma. Thus, we recently demonstrated that Ect2, Vav3, and Trio—3 GEFs that can activate Rac1—are necessary for glioblastoma cell invasion and are overexpressed in glioblastoma tissue versus low-grade glioma or nonneoplastic brain cells.[35] It would therefore be of great interest to perform similar studies in medulloblastomas.

Disclosure

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