The Cytoskeletal Adapter Protein Spinophilin Regulates Invadopodia Dynamics and Tumor Cell Invasion in Glioblastoma


Abstract

Glioblastoma is a primary brain cancer that is resistant to all treatment modalities. This resistance is due, in large part, to invasive cancer cells that disperse from the main tumor site, escape surgical resection, and contribute to recurrent secondary lesions. The adhesion and signaling mechanisms that drive glioblastoma cell invasion remain enigmatic, and as a result there are no effective anti-invasive clinical therapies. Here we have characterized a novel adhesion and signaling pathway comprised of the integrin αvβ8 and its intracellular binding partner, Spinophilin (Spn), which regulates glioblastoma cell invasion in the brain microenvironment. We show for the first time that Spn binds directly to the cytoplasmic domain of β8 integrin in glioblastoma cells. Genetically targeting Spn leads to enhanced invasive cell growth in preclinical models of glioblastoma. Spn regulates glioblastoma cell invasion by modulating the formation and dissolution of invadopodia. Spn-regulated invadopodia dynamics are dependent, in part, on proper spatiotemporal activation of the Rac1 GTPase. Glioblastoma cells that lack Spn showed diminished Rac1 activities, increased numbers of invadopodia, and enhanced extracellular matrix degradation. Collectively, these data identify Spn as a critical adhesion and signaling protein that is essential for modulating glioblastoma cell invasion in the brain microenvironment.

Implications: Tumor cell invasion is a major clinical obstacle in glioblastoma and this study identifies a new signaling pathway regulated by Spinophilin in invasive glioblastoma. Mol Cancer Res; 14(12); 1–11. ©2016 AACR.

Introduction

Glioblastoma is a rapidly progressive and highly invasive primary brain cancer that has a median survival time of less than two years after diagnosis (1). While a great deal is known about genes and pathways that promote glioblastoma initiation and growth, relatively little is understood about mechanisms that promote tumor cell invasion (2). Glioblastoma cells often exploit blood vessels and their ECM-rich basement membranes for selective growth and invasion in the brain microenvironment (3). Most cells interact with the ECM via integrins, a family of cell surface receptors consisting of α and β subunits (4). Integrin–ECM affinity is modulated via “inside-out” signaling mechanisms involving proteins such as talins and kindlins that bind to β integrin cytoplasmic domains and induce conformational changes in extracellular regions (5). ECM engagement subse-
RhoGDIs act in concert with guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP) to balance Rho protein activation. While other integrins stimulate Rho GTPases via interactions with GEFs, αββ integrin forms complexes with RhoCD11 to suppress activation of Rho GTPases (21). More recently, we have discovered that αββ integrin also forms complexes with the cytoplasmic tyrosine phosphatase PTP-PEST, and this complex dynamically regulates the phosphorylation status of RhoCD11 in migrating cells (22).

Spinophilin (Spn) is a 130-kDa scaffolding protein that contains protein phosphatase 1 (PP1), PDZ, and actin-binding domains (23) that is highly expressed in the brain where it regulates various signaling events to promote dendritic spine formation (24). Spn also modulates cytoskeletal dynamics, in part, via direct interactions with F-actin as well as α-actinin, a focal adhesion protein and actin regulatory factor (25). Spn is a regulatory subunit for PP1 and controls PP1 substrate specificity by subcellular localization (26). For example, in developing axons, Spn is reported to interact with doublecortin, leading to its dephosphorylation by PP1 and alterations in cytoskeletal dynamics (27). Recent reports have shown that downregulation of Spn gene expression promotes tumor cell growth and enhances metastasis in cancers of the colon, lung, and breast (28), although the exact signaling mechanisms that control these processes remain unclear.

Here, we have used biochemical and genetic strategies to identify Spn in glioblastoma cells as a binding partner for the ββ integrin cytoplasmic tail. We show that Spn is essential for the negative regulation of perivascular glioblastoma cell migration and invasion in the brain microenvironment. Genetic inhibition of Spn expression in mouse astrocytoma cells or human glioblastoma cells leads to significantly larger brain tumors due to enhanced invasive growth patterns. Finally, we show that Spn suppresses brain tumor cell invasion, in part, via control of Rac1 GTPase activities and invadopodia disassembly. Collectively, these results reveal new and important functions for Spn in regulating tumor cell–invasive growth in the brain via modulation of invadopodia dissolution.

Materials and Methods

Proteomics experiments

Detergent-soluble lysates (1 mg/mL) from primary human glioblastoma cells were used for immunoprecipitation experiments with control rabbit IgG or a rabbit polyclonal antibody directed against the ββ integrin cytoplasmic domain that has been described elsewhere (19). Antibody complexes were immobilized using Protein G-Sepharose. Immunoprecipitated proteins were resolved on an SDS-polyacrylamide gel under reducing conditions. Control and experimental bands were cut from the gel, and subjected to in-gel digestion with Trypsin. Peptides were extracted with 12-cm of reversed-phase Magic C18 packing material (5 μm, 200 Å; Michrom Biosources, Inc.). SEQUEST matches were filtered by XCorr scores to a less than 1% false discovery rate when the control proteins were eliminated and protein matches were required to have three spectral matches, no identifiable false positive peptides remained.

Experimental mice

Generation of Spn+/− mice used in this study has been detailed elsewhere (24). Spn+/− mice, maintained on a mixed C57Bl6/129S4/FVB mixed genetic background, were interbred to generate Spn−/−, Spn+/−, and Spn−/− littermates for experimental analyses. Genotypes were confirmed using genomic DNA isolated from ear tissue and PCR-based methodologies. Primer sequences used for genomic PCR reactions are as follows: 5′-GGAGACACTCCTGATTCCC-3′, 5′-GCCCTACAGATAGACAGAGG-3′ and 5′-AGG-TGAGATCGAAGAGATC-3′. PCR amplifies a wild-type product of 500 base pairs and mutant (Spn−/−) product of 750 base pairs, with both products being amplified in heterozygous (Spn+/−) animals. NCR-nu/nu mice were purchased from Jackson Laboratories and used for all experiments involving intracraniar injections of mouse astrocytoma cells and human glioblastoma cells. For brain isolation, adult mice were anesthetized and brains were fixed by cardiac perfusion with ice-cold 4% PFA/PBS.

Isolation and transformation of mouse astroglial cells

Astroglial progenitors were cultured from cerebral cortices of wild-type or Spn−/− littermates (P0) and propagated on laminin-coated dishes, as described previously (19). To generate retroviral stocks, we transfected 293T Phoenix cells with pLXSP-puro-E6/E7 and pLXSNeO-G12VH-Ras (29). Primary astroglial cells were transduced with the E6/E7 retroviral supernatant and cells were selected in growth media containing 1 mg/mL puromycin for 5 days. Puromycin-resistant clones were pooled and transduced with G12VH-Ras retrovirus supernatant, and selected for 10 days in G418 (900 μg/mL).

Human glioblastoma tissue samples and cultured cells

Approval for the use of human specimens was obtained from the Institutional Review Board at the University of Texas MD Anderson Cancer Center (Houston, TX). LN229 glioblastoma cells were purchased from ATCC. Cells were cultured in DMEM high-glucose sodium pyruvate supplemented with 1% penicillin/streptomycin and 10% FBS, at 37°C in an atmosphere with 5% CO₂. Primary human glioblastoma cells used for original identification of ββ integrin–Spn interactions were cultured from patient samples. Briefly, tumor tissue was dissociated by trituration with a polished glass pipette and cells were cultured in the following growth media: DMEM-F12 (Mediatech), 20 ng/mL EGF and bFGF (Biosource), B27 supplement (Gibco), and one unit per mL penicillin–streptomycin (Sigma-Aldrich).

Antibodies and plasmids

The anti-ββ integrin antibody used for immunoblotting has been described previously (12). The following antibodies were purchased: rabbit anti-Spn and anti-PP1 antibodies (Santa Cruz Biotechnology), chicken anti-Nestin (Neuromics), rat anti-mouse CD31 (BD Biosciences), rabbit anti-β-actin pAb (Sigma), rabbit anti-GFAP pAb (Dako), and anti-Ras and anti-E7 antibodies (Santa Cruz Biotechnology). The anti-myc mAb was purchased from Clontech Laboratories. Secondary antibodies used for imaging were conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes). Most sections were analyzed using a Zeiss Axio Imager.Z1 microscope. For Far Western blotting experiments, detergent-soluble lysates from LN229 cells expressing nontargeting control shRNAs or shRNAs targeting Spn were resolved on a 7.5% SDS gel and transferred to nitrocellulose.
membranes. Purified GST or GST-b8 integrin probes (5 μg/mL) were added to the membranes for 12 hours. Membranes were probed with an anti-GST antibody conjugated with HRP (Amer sham Biosciences) and membranes were analyzed by chemiluminescence.

pGIPZ lentiviral plasmids expressing various shRNAs were obtained from an institutional core facility via Dharmacon. The full-length human Spn cDNA was provided by Dr. Amacio Carnero. Myc-tagged human Spn cDNAs were generated by standard PCR-based methods. Constructs were cloned into the pcDNA3.1A plasmid for mammalian expression. For the Crispr/Cas9 experiments, we cloned gDNAs into the LentiCRISPR (pXPR-001) expression vector purchased from Addgene. Infected cells were selected in puromycin and Spn targeting was confirmed by immunoblotting and genomic analyses. gDNA sequences were selected using an open source design tool (http://crispr.mit.edu). The Spn gDNA sequences are as follows: 5’-ACTTTTGATGGTGTCGCTG-3’, 5’-GTCCGACCGGCGCCCAG-3’, 5’-GGATTACGATCGTCGCAACGGAGCGCCCCAG-3’, and 5’-GGCACGATGTAATCCGTG-3’.

Intracranial tumor cell implantation

All animal procedures were conducted under Institutional Animal Care and Use Committee–approved protocols. Nude mice were anesthetized and an automated micropump (Stoelting Instruments) was used to dispense cells in PBS. Astroglial progenitors cultured for less than four passages after transformation were used for the experiments. Nude mice were stereotactically injected with 1 × 10^5 Spn+/+ or Spn-/- transformed astroglial cells. Xenograft tumors were generated by stereotactically injecting 2 × 10^5 LN229 glioblastoma cells into immunocompromised mice.

Invadopodia assays and Rac1 activity FRET

LN229 control cells or cells expressing Spn gDNAs were plated on acid-washed coverslips coated with fluorescent gelatin and incubated for 6 hours. Subsequently, cells were fixed in 4% paraformaldehyde and stained for F-actin and cortactin to visualize invadopodia. LN229 cells expressing Cas9 or Spn gDNA/Cas9 and the Raichu-Rac1 single-channel FRET probe (4 × 10^6 cells) were plated on the fibronectin-coated (10 μg/mL) MatTek dishes with phenol red-free complete growth media. Cells were incubated at 37°C in an atmosphere with 5% CO2 for 20 hours prior to imaging. Time-lapse series images were acquired every minute for 30 minutes using a Zeiss LSM780 confocal microscope equipped with a Plan-Apo 40X/1.40NA oil objective. Ratio maps for visualization of Rac1 activity were generated using ImageJ. Briefly, stack of CFP and FRET images were first processed using a 3 × 3 median filter to remove background. Then, the median-filtered stacks were used to create a ratio stack the FRET channel divided by the CFP channel.

Statistical analysis

Student t test was performed to determine statistically significant differences between groups. The Wilcoxon rank-sum test was used for analysis of Kaplan–Meier survival results. Microsoft Excel (Microsoft) was used to calculate statistics.

Results

b8 integrin, which heterodimerizes exclusively with the αv integrin subunit (Fig. 1A), contains a cytoplasmic tail that is distinct from other integrin subunits. Alignment of the primary amino acid sequence reveals lack of conserved motifs, for example, NPXY motifs and juxtamembrane sequences, which are present in the other β subunits that heterodimerize with αv integrin. (Fig. 1B). We analyzed levels of cell surface αv-containing integrins using primary human glioblastoma cells and biotinylation/immunoprecipitation strategies. As shown in Fig. 1C, αvβ8 integrin is the major αv-containing integrin, with significantly lower levels of αvβ3 and αvβ5 integrin detected on the tumor cell surface. To identify intracellular signaling proteins that interact with the b8 integrin cytoplasmic tail, we performed coimmunoprecipitation experiments with anti-b8 integrin antibodies and primary human glioblastoma cells followed by mass spectrometry–based analyses. As shown in Fig. 1D and Supplementary Table S1, the major proteins that coimmunoprecipitate with b8 integrin include αv integrin and the scaffolding protein, Spinophilin/PPP1R9B. Interactions between b8 integrin and Spn were confirmed in pull-down assays using a GST-tagged fusion protein containing the entire cytoplasmic tail of b8 integrin (Fig. 1E). In addition, Spnb8 integrin protein interactions were verified by coimmunoprecipitation in LN229 glioblastoma cells (Fig. 1F). In addition to the biochemical interactions in cell lysates, we used Far Western blotting approaches to show direct protein–protein interactions between Spn and the b8 integrin cytoplasmic domain in vitro (Supplementary Fig. S1A). Spn and b8 integrin also colocalized in cultured LN229 glioblastoma cells, as revealed by immunofluorescence (Supplementary Fig. S1B).

To identify the region of the Spn protein that interacts with b8 integrin, we generated and expressed various recombinant myc-tagged protein constructs lacking select N-terminal and C-terminal regions (Fig. 1F–H; Supplementary Fig. S1C–S1F). The GST-b8 integrin fusion protein was used to test for interactions with myc-tagged Spn constructs expressed in Spn-/- astrocytes. As shown in Fig. 1I, the N-terminal region of Spn containing the receptor-binding domain (RBD) and the actin-binding domain (ABD) did not interact with b8 integrin. In contrast, the C-terminus of Spn protein, consisting of the PDZ domain and the coiled coil (CC) domain, is required for interactions with b8 integrin. The isolated CC domain did not show an interaction, identifying a requirement for the Spn PDZ domain, possibly in conjunction with the CC region, in mediating interactions with the b8 integrin cytoplasmic tail.

We next analyzed Spn protein expression levels in human glioblastoma cells and tissue samples. Anti-Spn immunoblots were performed with detergent-soluble lysates from six different human glioblastoma cell lines, revealing robust Spn expression in LN229 cells (Fig. 2A). Lower levels of Spn protein were detected in SNB19 and U87 glioblastoma cells, and undetectable Spn levels were found in LN18, U373, and LN428 glioblastoma cells. In addition, anti-Spn antibodies were used to immunoblot lysates from 12 different freshly resected human glioblastoma samples. Robust levels of Spn were detected in some tumor samples and low or undetectable levels were present in other tumors (Fig. 2B). Given that Spn is a regulatory subunit for PP1, we also monitored PP1 protein levels in cell lines and tumor lysates. As shown in Fig. 2A and B, PP1 was detected in all glioblastoma cells and most tumor lysates analyzed by anti-PP1 immunoblotting. PP1 can function independently of Spn; hence, this likely explains why PP1 and Spn expression does not entirely overlap in the various glioblastoma samples. To analyze spatial patterns of Spn protein in tissue samples, we next used IHC with anti-Spn antibodies. As
Spinophilin binds to the cytoplasmic tail of β8 integrin. A, The αv subfamily of integrins consists of five members. Note that β8 integrin dimerizes exclusively with the αv subunit. B, Alignment of primary amino acid sequences from the five β integrin subunits that pair with αv integrin. Note that the β8 integrin cytoplasmic tail does not share common motifs with other integrin subunits. C, αvβ8 integrin is the major αv-containing integrin on the surface of primary glioblastoma (GBM) cells, as revealed by cell surface biotinylation and immunoprecipitation. Antibodies used for immunoprecipitation are indicated at the bottom of the image. D, Primary human glioblastoma cells were immunoprecipitated with control IgG or an anti-β8 integrin antibody and gels were silver stained. Trypsin-digested bands were identified by mass spectrometry. Note that αv integrin and Spn are the major proteins that coimmunoprecipitated with β8 integrin. E, GST or GST fused to the cytoplasmic domain of β8 integrin (GST-β8cyto) were purified from bacteria and used as probes in GSC lysates to confirm interactions between GST-β8cyto and Spn. F, Spn and β8 integrin interact in LN229 glioblastoma cell lysates as determined by coimmunoprecipitation. G and H, Schematic diagram (G) and immunoblot (H) showing domain structure and full-length myc-tagged Spn protein and various deletion constructs expressed in HEK-293T cells. I, GST or GST-β8cyto proteins were purified from bacteria and used as probes in lysates from Spn+/- mouse astrocytes forcibly expressing full-length Spn or various deletion constructs. Note that full-length Spn and the deletion construct containing the PDZ/CC region interacts with β8 integrin.

Abbreviations: ABD, actin binding domain; RBD, receptor binding domain; PP1, protein phosphatase 1 binding domain; CC, coiled coil domain.

shown in Fig. 2C, Spn protein was diffusely expressed in neural cells and blood vessels in the normal brain. In human glioblastoma sections, Spn was expressed mostly in tumor cells as well as in some intratumoral capillaries, although glomeruloid-like blood vessels, which develop via microvascular cell hyperproliferation and are a defining feature of glioblastoma, were largely negative for Spn expression (Fig. 2D). In addition to the primary glioblastoma specimens, we analyzed Spn expression by immunofluorescence in recurrent glioblastoma samples taken from patients after radiation and chemotherapy. Spn protein expression was detected at robust levels primarily within glioblastoma cells of these recurrent tumor samples (Supplementary Fig. S2).

Querying the open source The Cancer Genome Atlas (TCGA) database for human glioblastoma (http://cabi.ltdk.helsinki.fi/ anduril/tcga-gbm) revealed that Spn/PPP1R9B mRNA expression in tumors was diminished by approximately 50%, with a median expression fold-change of 0.527 in glioblastoma samples versus noncancerous control brain tissue (data not shown). Analysis of data in the Ivy GBM Atlas Project (GAP) (http://glioblastoma.alleninstitute.org/maseq/search/index.html) revealed that Spn mRNA was heterogeneously expressed in different tumor regions. Most notably, Spn mRNA is enriched as well as at the margins of tumor samples and in some infiltrative tumor regions, but was expressed at significantly lower levels in core tumor regions and in intratumoral regions containing microvascular proliferation (Supplementary Fig. S3A), which is consistent with the IHC results in Fig. 2. In glioblastoma there are at least four molecular subtypes based on genomic alterations and gene expression profiles (30). However, querying the Ivy GAP database revealed Spn mRNA was differentially expressed in tumor regions, regardless of classification by glioblastoma molecular subtype (Supplementary Fig. S3B), although Spn levels were notably higher in classical and neural subtypes. Segregating tumor samples based on relative Spn mRNA expression levels (Spnhigh vs. Spnlow) did not reveal statistically significant differences in overall patient survival (Supplementary Fig. S3C).

Next, to determine the roles for Spn in brain tumor initiation, growth, and invasion in vivo, we developed mosaic mouse models of glioblastoma. Retroviral-delivered oncogenes were used to sequentially immortalize and transform primary mouse astroglial cells as we have previously reported (16; Supplementary Fig. S4A and S4B). Cells were immortalized with E6/E7 proteins and transformed using a H-Ras-V12G oncogene. E6/E7 proteins inhibit the tumor suppressors p53 and Rb, which are commonly inhibited in glioblastoma.
inactivated in glioblastoma. Hyperactivation of the Ras pathway via EGFR amplification or mutation is linked to glioblastoma progression, and expression of oncogenic Ras in mice leads to spontaneous brain tumorigenesis (31). Transformed astrocytes generate intracranial tumors in vivo that displayed many histologic features of grade III anaplastic astrocytoma (Supplementary Fig. S4C), and some tumors progress to glioblastoma-like status (data not shown).

Analysis of Spn protein expression in primary mouse astroglial cells isolated from wild-type mouse pups revealed enrichment at the cell membrane and colocalization with F-actin, whereas Spn protein was absent in Spn−/− cells owing to gene ablation (Fig. 3A–E). Expression of retroviral-delivered oncoproteins was confirmed in wild-type and Spn−/− mouse astrocytes (Fig. 3F). We intracranially injected wild-type and Spn−/− transformed mouse astrocytes into immunocompromised adult mice (n = 8 mice per cell type). Although all mice were sacrificed at two months postinjection, animals injected with Spn−/− cells showed more obvious tumor-related neurologic deficits including a hunched posture, ataxia, and seizures (data not shown). Brain tumor growth and invasion were analyzed in coronal brain slices. As shown in Fig. 3G, in comparison with wild-type–transformed astrocytes, Spn−/−-transformed cells generated significantly larger intracranial tumors. Microscopic analysis of hematoxylin and eosin (H&E)-stained brain sections confirmed that Spn−/− tumors were significantly larger in relation to tumors derived from wild-type cells (Fig. 4A–D). Spn−/− astrocytes generated tumors with increased invasive properties, and clusters of tumor cells often showed perivascular dispersal patterns away from the primary mass with secondary lesions forming at the pial surface of the brain (Fig. 4C and D). Immunofluorescence staining revealed that wild-type and Spn−/−-transformed astrocytes expressed laminin and nestin within the brain microenvironment, and tumors were well vascularized, as revealed by anti-CD31 immunofluorescence (Fig. 5A–D).

In addition to the mosaic mouse models, we analyzed functions for Spn in LN229 human glioblastoma cells, which express high levels of endogenous Spn protein (Fig. 2A). Lentiviral-delivered shRNAs were used to target the Spn gene, which led to the diminished expression of Spn mRNA (data not shown) and protein (Supplementary Fig. S5A). Intracranial implantation of LN229 cells expressing nontargeting control shRNAs or shRNAs targeting Spn revealed important roles in tumor growth and invasion in the brain. In comparison with animals injected with LN229 cells expressing control shRNAs, mice injected with cells expressing Spn shRNAs developed earlier and more obvious tumor-related neurologic deficits (data not shown). Microscopic analysis revealed that LN229 tumors lacking Spn were significantly larger (Supplementary Fig. S5B–S5E), and often grew to comprise nearly the entire brain hemisphere. Microscopical analysis of H&E-stained samples confirmed larger tumor volumes and enhanced invasion in LN229 tumors expressing...
Spn shRNAs, as compared with tumors derived from cells expressing control shRNAs (Supplementary Fig. S6). We confirmed continued stable downregulation of Spn protein expression in vivo by immunofluorescently labeling LN229 brain tumor sections with anti-Spn antibodies (data not shown). Immunofluorescence and immunohistochemical microscopic analyses revealed enhanced invasion in tumors formed from LN229 cells expressing Spn shRNAs (Fig. 5E–H; Supplementary Fig. S7). Collectively, data from both the mosaic mouse models and LN229 xenograft models reveal important and novel

Figure 3.
Genetically targeting Spn in a mosaic model of brain cancer enhances invasive tumor cell growth. A–D, Primary astrocytes were cultured from the cerebral cortices of wild-type (A, C) or Spn−/− (B and D) neonatal mice. Adherent cells were immunolabeled with anti-Spn (red) combined with anti-paxillin (green) to visualize focal adhesions (A, B) or phalloidin-FITC (green) to visualize the actin cytoskeleton (C and D). E, Lysates from primary astrocytes isolated from wild-type or Spn−/− pups were immunoblotted with anti-Spn. F, Lysates from primary, immortalized, and transformed mouse brain astrocytes were immunoblotted with anti-Ras or anti-E7 antibodies. G, Images of three different brains harboring tumors derived from wild-type (top) or Spn−/−-transformed mouse astrocytes (bottom). Note that Spn−/− tumors are significantly larger than control tumors, and indicated by dashed lines.

Figure 4.
Spn−/− mosaic mouse models display enhanced invasive growth in vivo. A–D, Brains of mice harboring tumors generated from wild-type (A, B) or Spn−/− (C and D) transformed mouse astrocytes were sliced coronally, embedded in paraffin, and sections were stained with H&E. Note that tumors derived from Spn−/−-transformed cells are significantly larger than wild-type tumors. Spn−/− cells show more robust invasion away from the primary mass, often reaching the pial surface of the brain (arrows in D). Scale bars, 50 μm.
functions for Spn in negatively regulating tumor cell growth and invasion in the brain microenvironment.

A critical step in the invasion process is the formation of invadopodia, which are membrane protrusions enriched in F-actin, the actin-binding protein cortactin, and other signaling effectors (32). Invadopodia are utilized by cancer cells to survey the surrounding microenvironment to initiate and sustain proinvasive signaling cascades. Therefore, we analyzed Spn-dependent invadopodia formation in glioblastoma cells on FITC-gelatin (Supplementary Fig. S8). As the lentiviral-delivered shRNAs targeting Spn did not yield a complete inhibition of gene expression (Supplementary Fig. S5A), we utilized Crispr/Cas9 gene editing methodologies to more effectively block Spn gene expression. In comparison with control cells, LN229 cells that lack Spn contain increased numbers of actin-expressing and cortactin-expressing invadopodia (Fig. 6A and B). Cells expressing Spn gDNAs also displayed a significant increase in the degradation of FITC-gelatin (Fig. 6C) and showed diminished peripheral actin levels, likely owing to increased actin in invadopodia (Fig. 6D). As shown in Fig. 6E, we obtained a near complete loss of Spn protein expression using lentiviruses expressing Cas9 and different gDNAs targeting various regions of the Spn gene. Loss of Spn gene expression in LN229 cells also led to increased proliferation (Fig. 6F) and migration in scratch-wound assays (Fig. 6G).

The results in Fig. 6 reveal that while Spn is dispensable for the formation of invadopodia in glioblastoma cells, it likely has important roles in the dynamic maintenance and/or dissolution of invadopodia structures. Various reports have shown that adhesion-mediated activation of members of the Rho family of small GTPases, comprised of RhoA, Cdc42, and Rac1, is essential for invadopodia dynamics and cell invasion (33). In particular, proper spatiotemporal regulation of Rac1 by the TrioGEF promotes invadopodia disassembly and cell invasion (34). Spn interacts with various GEFs (35) to control F-actin dynamics in the nervous system. To determine whether Spn regulates Rac1 activation, we utilized the Raichu-Rac1 fluorescent biosensor (36) to monitor levels of Rac1 activities modulated by GEFs and GAPs. Raichu-Rac1 consists of truncated Rho sequences fused to the Rac-interactive binding domain (CRIB) of Pak1 flanked by YFP at the N-terminus and CFP at the C-terminus. Intramolecular interactions between GTP-bound Rac1 and CRIB juxtapose YFP and CFP, leading to Förster resonance energy transfer (FRET) from CFP to YFP. As shown in Fig. 7A and B, significantly lower levels of GTP-bound Rac1 protein are detected in LN229 cells that lack Spn. Time-lapse imaging studies confirmed the diminished levels of Raichu-Rac1 FRET in LN229 glioblastoma cells lacking Spn expression (Supplementary Videos S1 and S2). On the basis of these data, we propose a model in which Spn recruits GEFs to locally active Rac1 (and possibly other Rho GTPases) to promote invadopodia maintenance and disassembly. Tumor cells lacking Spn form invadopodia, but do not normally maintain and disassemble these structures, leading to increased numbers of invadopodia per cell, enhanced ECM degradation, and more robust cell invasion in the brain microenvironment (Fig. 7C).

Discussion

Here, we have characterized mechanisms by which the intracellular scaffolding protein Spn regulates invasive cell growth in the malignant brain cancer glioblastoma. We report the following novel findings: (i) the C-terminus of Spn binds directly to the β8 integrin cytoplasmic tail (Fig. 1), (ii) Spn is expressed in human glioblastoma cells in vitro and in tumor samples in situ (Fig. 2), (iii) Spn suppresses perivascular tumor cell invasion in preclinical mouse models of glioblastoma (Figs. 3–5), (iv) Spn modulates invadopodia dynamics and disassembly in glioblastoma cells (Fig. 6), and (v) Spn promotes the activation status of the Rac1 GTPase to regulate invadopodia dynamics (Fig. 7). Collectively, these data identify Spn as a critical intracellular scaffolding
protein that controls invasion by negatively regulating glioblastoma cell invadopodia dynamics.

In studies of cancers outside of the brain, diminished levels of Spn have been reported to correlate with enhanced malignancy. For example, targeting Spn gene expression in breast tumor cells using RNAi-mediated methodologies increases self-renewal of stem cell populations and enhances migration and metastasis to the lung (37, 38). Similar findings have been reported in primary tumors of the colon (39, 40). Reflecting the multi-domain composition of Spn and its scaffolding and signaling roles, the mechanisms by which Spn loss promotes malignancy are complex. For example, down-regulation of Spn leads to differential gene expression in breast cancer cells and alters PP1 sub-cellular localization and specificity for substrates including retinoblastoma and other proteins (39, 41). TCGA data reveal that Spn expression is diminished in glioblastoma versus non-cancerous brain samples, and our experimental results show that Spn down-regulation in glioblastoma cells causes enhanced invasive growth.

Figure 6.
Spn regulates invadopodia dynamics to promote glioblastoma (GBM) cell invasive growth. A and B, Control LN229 cells (A) or cells expressing Spn gDNAs (B) were analyzed on coverslips coated with FITC-gelatin for invadopodia formation. Note that cells lacking Spn contain increased numbers of invadopodia, as evidenced by actin and cortactin expression/localization and FITC-gelatin degradation. C and D, Quantitation of Spn-dependent FITC-gelatin degradation (C) and actin puncta (D) in LN229 cells. The degradation index was calculated as the ratio of degradation area/actin area per field (n = 8–10 fields/condition), †, P < 0.05. Box and whisker plots show distribution of actin in invadopodia defined as actin colocalized with cortactin. The results summarize at least 320 actin-rich puncta per condition, †, P < 0.05. E, Crisp/Cas9-mediated gene editing was used to target Spn in LN229 cells. Note the absence of Spn protein as revealed by immunoblotting detergent-soluble lysates. F and G, LN229 cells expressing Cas9 and control gDNAs or Cas9 and gDNAs targeting Spn were assayed for proliferation (F) and migration (G). In comparison with control cells, LN229 cells expressing Cas9 and gDNAs targeting Spn display enhanced migration in vitro in scratch-wound assays.

Figure 7.
Spn promotes Rac1 GTPase activation in glioblastoma (GBM) cells. A, LN229 cells infected with control lentivirus or lentivirus expressing gDNAs targeting Spn were transiently transfected with a plasmid expressing the Rac1-Raichu FRET biosensor. Shown are representative FRET/CFP ratio images taken from a confocal time-lapse series. Images are presented using an intensity modulated display to associate intensity (activity) with color and hue (red = high activity; blue = low activity). Scale bar = 10 μm. B, Quantitative analysis of FRET activity (mean ± SEM, ***; P < 0.001) in arbitrary units. FRET ratios were collected for each cell in 2–4 fields (2–4 cells per field) for every time point for analysis (n = 1,147). Experiments were done in triplicate. C, A model for Spn regulation of invadopodia dynamics in glioblastoma cells. Spn normally suppresses glioblastoma cell invasion by promoting Rac1 activation, which leads to dissolution of invadopodia and diminished invasion. Loss of Spn leads to increased numbers of invadopodia and enhanced tumor cell invasion, which is supported by our in vitro and in vivo data. αvβ8 integrin interacts directly with Spn, which likely regulates invadopodia formation and disassembly. Other cell surface receptors and intracellular proteins also bind to Spn and likely control its functions independently of αvβ8 integrin. Furthermore, in addition to Rac1 Spn interacts with additional signaling effectors, such as PPI, to modulate invadopodia dynamics.
in the brain microenvironment. However, glioblastoma patients with relative low versus high levels of Spn expression do not show differences in overall survival, revealing that Spn alone is not an accurate biomarker to predict outcome. It will be interesting to determine if combining Spn and additional genes to generate an invasive “gene signature” will better predict patient prognosis or response to therapy.

The C-terminal domain of Spn, which contains the CC and PDZ domains, interacts with the β8 integrin cytoplasmic tail, suggesting that Spn oligomerization, which is mediated by the CC domain (42), is important for integrin binding and/or signaling. The Spn C-terminus contains a type 5 PDZ domain, which interacts with short class 1 and class 2 PDZ binding peptide sequences (43). Interestingly, amino acids 713-716 within the cytoplasmic tail of human β8 integrin comprise an SNKI peptide sequence that is similar to SVKI, which is the class 2 PDZ peptide-binding motif. Hence, we postulate that the PDZ domain mediates interactions with β8 integrin cytoplasmic sequence, enabling the N-terminal regions of Spn to interact with additional effector proteins, including Fascin and PPL. Subsequent ECM engagement and outside-in signaling events likely dependent on Spn oligomerization via the CC domain. Given its roles in mediating activation of latent TGFβ, it is likely that these are involved in β8 integrin signaling via Spn. It is also possible that Spn may be involved in inside-out activation of αβ8 integrin, thus modulating integrin affinity for ECM protein ligands. Inside-out integrin signaling commonly occurs via interactions with intracellular scaffolding proteins such as talins and kindlins (44). However, β8 integrin lacks cytoplasmic tail NPYX binding motifs found in β1A, β2, β3A, β5, and β6 integrins that commonly recruit FERM domain-containing effector proteins that promote activation and signaling. In addition, the intracellular juxtamembrane region of β8 integrin is divergent from other integrin subunits. This region is involved in a “handshake” with the adjacent α integrin subunit to maintain the extracellular region in an inactive state (45). This juxtamembrane handshake is disrupted upon interactions between the β subunit and talins and kindlins. Furthermore, the β8 integrin extracellular region lacks a “deadbolt” domain that facilitates inside-out activation in other integrins (45), suggesting constitutive activation and ECM ligand engagement independently of classical integrin “inside-out” activation mechanisms.

A prior report used proteomics-based methods to identify multiple proteins that coimmunoprecipitate with Spn in the rodent brain (25), but in that study β8 integrin was not reported as a Spn-associated protein. Here, we have utilized an antibody directed against the β8 integrin cytoplasmic domain and a GST fusion protein containing the β8 integrin cytoplasmic tail to identify and validate Spn interactions in glioblastoma cells. Interestingly, anti-Spn antibodies we have tested did not coimmunoprecipitate β8 integrin from cells, suggesting that the Spn protein pool that interacts with β8 integrin is not recognized by these antibodies, or that the β8 integrin–Spn interaction masks the anti-Spn antibody epitopes. In the proteomics screen mentioned above, α-actinin (Actn1) was identified as a major Spn-interacting protein in the mouse brain. Indeed, Actn1 and Actn4 were identified in our proteomic experiments as β8 integrin-interacting proteins, but we have not detected interactions between Actn1/4 and β8 integrin by Far Western blotting (data not shown), suggesting that its interaction with β8 integrin is likely the result of Spn associations. Indeed, we have confirmed interactions between Spn and Actn1 by coimmunoprecipitation and colocalization at glioblastoma cell–ECM contacts, likely nascent and/or focal adhesions (data not shown). Genetically deleting β8 integrin expression, or truncating its cytoplasmic signaling domain to abrogate signaling, leads to defects in glioblastoma cell migration and invasion (17, 21). However, in this report, we show that tumor cells that lack Spn, a β8 integrin effector protein, display enhanced invasiveness. Therefore, we propose that β8 integrin balances proinvasive and anti-invasive signal transduction pathways. In the case of the Spn signaling cascade, β8 integrin serves to localize Spn to the cell’s leading edge to promote invadopodia disassembly and negatively regulate invasive signaling events. In the absence of Spn, this balance is perturbed, and β8 integrin continues to signal via other proinvasive effectors, such as the tyrosine phosphatase PTP-PEST. In addition, Spn in part likely modulates some intracellular signaling events independently of β8 integrin that impact glioblastoma cell growth and invasion.

Finally, understanding the signaling mechanisms that drive perivascular invasion in glioblastoma is not only fundamentally important but also clinically relevant. Brain tumor cells that disperse into healthy brain tissue escape surgical resection, remain largely resistant to chemotherapy and radiation, and invariably give rise to recurrent lesions (46). The significance of glioblastoma cell invasion has also been underscored by recent clinical trials with the VEGF-neutralizing antibody bevacizumab (47). Patients treated with bevacizumab showed short-term improvements in progression-free survival; however, overall survival rates have not improved due to inevitable tumor recurrence. Unexpectedly, about 40% of patients display unusually robust patterns of invasive growth and lethal secondary lesions (48). Furthermore, in preclinical models of breast cancer and pancreatic cancer small-molecule inhibitors of VEGF receptors have also been reported to enhance invasion and metastasis (49). Tumor recurrence and enhanced invasion following bevacizumab therapy has been linked, in part, to deregulation of the c-Met signaling pathway (50), although other intracellular signaling events are likely involved. On the basis of the data that we report here, it is intriguing to speculate that anti-VEGF therapies may lead to increased invasive growth in glioblastoma, or enhanced metastasis in non-brain tumors, via modulating Spn expression and related signaling effectors in cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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