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ORIGINAL ARTICLE

Autocrine semaphorin 3A signaling promotes glioblastoma dispersal

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Glioblastoma multiforme (GBM) is the most malignant glioma type with diffuse borders due to extensive tumor cell infiltration. Therefore, understanding the mechanism of GBM cell dispersal is critical for developing effective therapies to limit infiltration. We identified neuropilin-1 as a mediator of cancer cell invasion by a functional proteomic screen and showed its role in GBM cells. Neuropilin-1 is a receptor for semaphorin3A (Sema3A), a secreted chemorepellent that facilitates axon guidance during neural development. Although neuropilin-1 expression in GBMs was previously shown, its role as a Sema3A receptor remained elusive. Using fluorophore-assisted light inactivation and RNA interference , we showed that neuropilin-1 is required for GBM cell migration. We also showed that GBM cells secrete Sema3A endogenously, and RNA interference-mediated downregulation of Sema3A inhibits migration and alters cell morphology that is dependent on Rac1 activity. Sema3A depletion also reduces dispersal, which is recovered by supplying Sema3A exogenously. Extracellular application of Sema3A decreases cell-substrate adhesion in a neuropilin-1-dependent manner. Using immunohistochemistry, we showed that Sema3A is overexpressed in a subset of human GBMs compared with the non-neoplastic brain. Together, these findings implicate Sema3A as an autocrine signal for neuropilin-1 to promote GBM dispersal by modulating substrate adhesion and suggest that targeting Sema3A-neuropilin-1 signaling may limit GBM infiltration.
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Introduction

Malignant gliomas are highly invasive tumors with diffuse tumor borders. Glioblastoma multiforme (GBM) is the most common and aggressive type of gliomas with a mean survival of 1 year after diagnosis (Holland, 2001). A major obstacle in treating GBMs is the extensive tumor cell infiltration into the surrounding brain. Despite tumor resection and combined therapy, recurrence occurs in the vicinity of the resection margin due to individual cells that dispersed out of the primary tumor (Berens and Giese, 1999). Therefore, understanding the mechanisms that contribute to GBM cell dispersal is crucial for developing effective therapies.

Glioblastoma multiforme cell dispersal into the permissive brain environment occurs through activation of cell motility and invasion programs, some of which are shared with the mechanisms involved in neural development. For example, malignant gliomas have unique features in their infiltration process, in which the tumor cells preferentially move along established tracts such as vascular basement membranes and myelinated axons (Hoelzinger *et al*., 2007). Axon guidance molecules such as ephrin-B3 (Nakada *et al*., 2006) and Slit2 (Mertsch *et al*., 2008) and their receptors (Nakada *et al*., 2004) have been implicated in the invasive behavior of gliomas, suggesting that GBM dispersal may involve the activation of dormant cell motility programs used during neural development.

We have previously established functional proteomic screens targeting the surface proteome of invasive cancer cells (Eustace *et al*., 2004; Sloan *et al*., 2004) to identify novel molecules required for invasion. In this study, we describe the identification of neuropilin-1, a receptor for the semaphorin family of axon guidance molecules, as pro-migratory and address its role in GBM cell migration. Semaphorin3A (Sema3A) is the founding member of this family and acts as a repulsive signal for several types of developing neurons (Tran *et al*., 2007). On binding to neuropilin-1 and its co-receptor PlexinA1, Sema3A activates a signal transduction cascade that controls F-actin dynamics through activation of small GTPase Rac1 (Kolodkin *et al*., 1997; Turner *et al*., 2004). Although the role of neuropilin-1 as a Sema3A receptor is well characterized in the developing nervous system, its role in GBMs is not well established. In this study, we address the role of Sema3A in GBM cell migration and dispersal, two features that contribute to the aggressive phenotype of human GBMs.

Results

Functional proteomic screen identifies neuropilin-1 as a mediator of cancer cell invasion

To identify novel mediators of cancer cell invasion, we previously established a high-throughput functional

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proteomic screen targeting the surface proteome of HT1080 fibrosarcoma cells by fluorophore-assisted light inactivation (FALI) (Figure 1a) (Sloan *et al*., 2004). In FALI, a protein is transiently inactivated by fluorescein isothiocyanate (FITC)-labeled antibodies that generate short-lived reactive oxygen species on light irradiation (Beck *et al*., 2002). We generated a recombinant single-chain variable fragment (ScFv) antibody library targeting the HT1080 cell surface by phage display. These ScFvs were conjugated to FITC and used to inactivate their target proteins by FALI. The FALI-treated and matched unirradiated cells were then compared in a transwell invasion assay. The targets of ScFvs that significantly changed invasion after FALI were identified by immunoprecipitation and mass spectrometry (Sloan *et al*., 2004). We further investigated one ScFv (5C2) that caused the largest reduction in invasion after FALI ($56 \pm 9\%$) (Figure 1b). The reduction in invasion with 5C2 was even greater than the effect observed with a positive-control antibody targeting β1-integrin ($39 \pm 4\%$), a cell surface protein that mediates invasion (Guo and Giancotti, 2004). Light irradiation in the absence of an antibody caused no significant change in invasion confirming the specificity of FALI using 5C2.

We identified the target antigen bound by 5C2 by immunoprecipitation and mass spectrometry. The immunoprecipitation by 5C2 ScFv yielded a predominant band at 130 kDa with silver staining when separated on an SDS–polyacrylamide gel electrophoresis (data not shown). This band was analysed by matrix-assisted laser desorption/ionization mass spectrometry and identified as neuropilin-1 (SwissProt, O14786, http://ca.expasy.org/sprot) with 17 matching peptides covering 22% of the protein (206/923 residues) (Figure 1c). Three peptides (fragments 659–672, 680–782 and 776–787) identified neuropilin-1 in its full-length form as opposed to a neuropilin-1 splice variant that lacks amino acids 645–923 (Rossignol *et al*., 2000).

Inhibition of neuropilin-1 decreases GBM cell migration

Proteomic identification of neuropilin-1 as a pro-invasive protein prompted us to study its role in GBMs, the most invasive form of gliomas. The expression of neuropilin-1 has been observed previously in several cancers, including GBMs, and its high expression is correlated with poor prognosis (Kawakami *et al*., 2002; Osada *et al*., 2004; Kreuter *et al*., 2006). We assessed neuropilin-1 expression in human GBMs by immunohistochemistry and quantitative reverse transcriptase–PCR and found

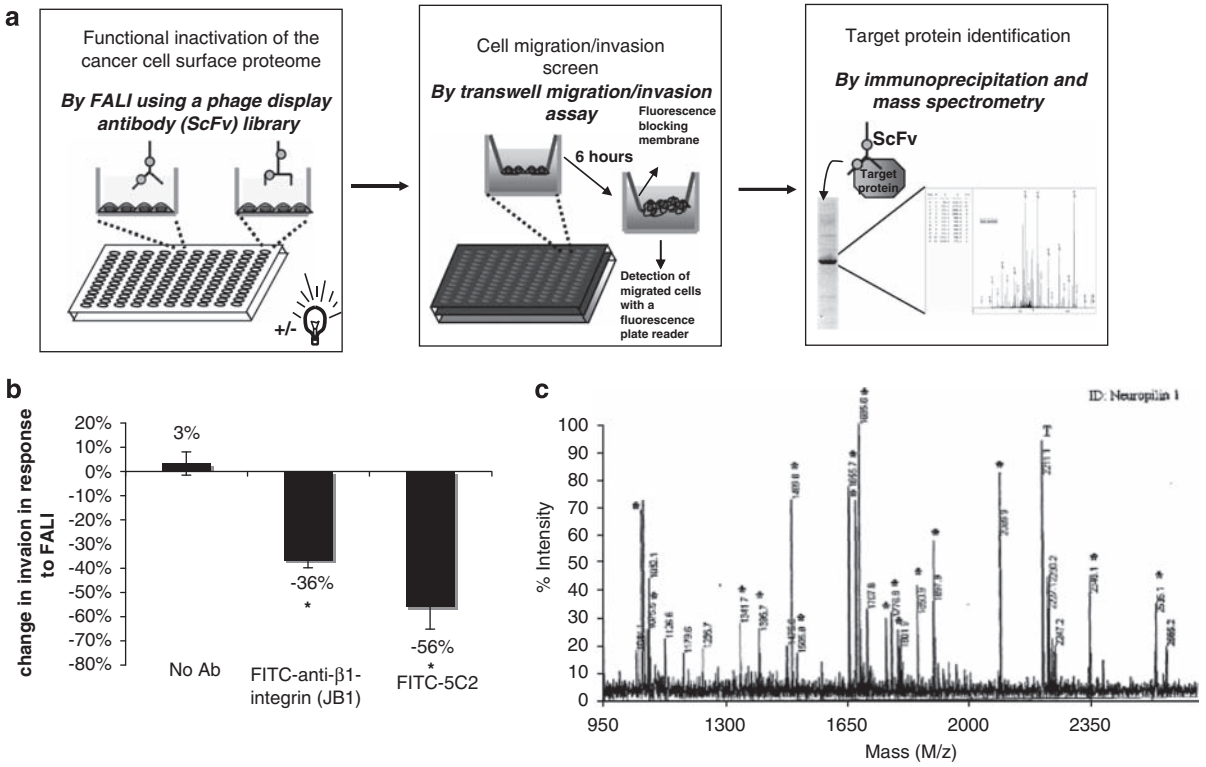


Figure 1 Functional proteomic screen identifies neuropilin-1 as a mediator of cancer cell invasion. (**a**) Overview of the proteomic screen. The 338 recombinant single-chain antibodies (ScFvs), a subgroup of a cell surface library generated by phage display, were coupled to fluorescein isothiocyanate (FITC) and used for fluorophore-assisted light inactivation (FALI). HT1080 cells either untreated or treated with FALI were then tested in a fluorometric transwell invasion assay. The ScFvs that caused a significant change in invasion after FALI were used to immunoprecipitate their target proteins, which were then identified by mass spectrometry. (**b**) The effect of FALI with 5C2, a neuropilin-1 binding ScFv, on HT1080 fibrosarcoma cell invasion. Shown here are the effects of no antibody negative control ($3 \pm 5\%$, ns), anti-β1-integrin-positive control ($36 \pm 4\%$, $P = 3.5\text{E-}17$, *t*-test) and 5C2 ($56 \pm 9\%$, $P = 0.0016$, *t*-test). (**c**) Identification of 5C2 target protein as neuropilin-1. Mass spectrum generated by matrix-assisted laser desorption/ionization analysis of tryptic digest of immunoprecipitated 130 kDa band. Peaks with * denote the 17 matching peptides in the neuropilin-1 sequence (SwissProt, O14786).

that neuropilin-1 is significantly overexpressed in GBMs (Supplementary Figure 1). These data, together with our proteomic identification of neuropilin-1 as a pro-invasive protein, suggest that neuropilin-1 could contribute to GBM dispersal. To test the role of neuropilin-1 *in vitro*, we chose GBM cell lines with varying levels of neuropilin-1 expression (Rieger *et al*., 2003; Hu *et al*., 2007). As shown by immunoblotting, the levels of neuropilin-1 were different across cell lines: U87MG (high), A172 (moderate), U251 (low) (human umbilical vein endothelial cell, an endothelial cell line, served as positive control) (Figure 2a).

We noted that the basal migration capacity of the three GBM lines correlated with their endogenous neuropilin-1 expression, where U251 cells have low, A172 cells have moderate and U87MG cells have high migration (data not shown). To specifically test the role of neuropilin-1 in GBM cell migration, we carried out FALI on GBM cells using FITC-conjugated 5C2. As 5C2 was initially selected on HT1080 cells, we tested whether 5C2 recognizes neuropilin-1 at the GBM cell surface. Using immunofluorescence, we verified that 5C2 binds to GBM cells, and the staining pattern is consistent with the endogenous neuropilin-1 levels. The A172 and U87MG cells displayed positive staining compared with the U251 cells (Figure 2b). We then tested the effect of FALI on neuropilin-1 by immunoblotting FALI-treated cell lysates for neuropilin-1. FALI of neuropilin-1 using FITC-5C2 caused a reduction in neuropilin-1, an effect not observed with light irradiation of FITC-IgG-treated samples (Figure 2c). Using FITC-5C2, the transwell migration of A172 cells decreased by 36% with FALI (Figure 2d). This was comparable to our positive-control CD44 (42%), a cell surface protein that promotes GBM cell migration (Okada *et al*., 1996). FALI with FITC-IgG, a non-specific antibody control, did not significantly affect migration. The effect of FALI of neuropilin-1 on U87MG cells was even greater (51%) (Figure 2e). U251 cells, with low neuropilin-1 expression, did not respond to the FALI of neuropilin-1 (data not shown). To further test the role of neuropilin-1, we used a neuropilin-1 function-blocking antibody and RNA interference (RNAi). Cells that were treated with anti-Nrp-1 migrated significantly less than controls. The reduction in migration was comparable to the levels observed with FALI (30%) (Figure 2f). Transfection of cells with neuropilin-1-specific small interfering RNA (siRNA) (Nrp-1 siRNA) caused a significant reduction in protein levels ($>90\%$) as assessed by immunoblotting and neuropilin-1-depleted cells migrated significantly less (33%) than controls (Figure 2g). Taken together, these results implicate neuropilin-1 in GBM cell migration *in vitro*.

Sema3A promotes GBM cell migration

Neuropilin-1 can influence cell migration in response to distinct ligands (Roush, 1998). Sema3A and vascular endothelial growth factor (VEGF165) are the major ones with roles in axonal chemorepulsion and endothelial cell migration, respectively (Klagsbrun *et al*., 2002). To test whether neuropilin-1 on GBM cells would respond to these ligands, we treated GBM cells with Sema3A or VEGF165 and assessed their migration. VEGF165 had no effect on GBM migration, but was functionally active as it enhanced human umbilical vein endothelial cell migration (Supplementary Figure 2). In contrast, Sema3A treatment caused a significant increase in migration of GBM cells (Figure 3a). The Sema3A-induced increases in migration correlated with the endogenous levels of neuropilin-1 in GBM cells (U87MG: 51%; A172: 25%; U251: 4%, nonsignificant). Taken together, our findings suggest that neuropilin-1 responds primarily to Sema3A, and not to VEGF165, to enhance GBM cell migration.

To examine whether GBM cells express Sema3A endogenously, we carried out immunoblotting on the cell lysates and conditioned media. This analysis showed that GBM cells express Sema3A and secrete it into the medium (Figure 3b). To test the role of endogenously expressed Sema3A in GBM cell migration, we transfected GBM cells with Sema3A siRNA, which resulted in marked downregulation of Sema3A protein (80%) as assessed by immunoblotting of the conditioned media (Figure 3c). The reduction in Sema3A mRNA levels was also verified by quantitative reverse transcriptase–PCR (data not shown). To rule out the effects of cell death, we checked the viability of cells by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, which remained unchanged on Sema3A inhibition (Figure 3c). Compared with control cells, Sema3A-depleted cells migrated significantly less. The reduction in migration with Sema3A depletion was 48% for A172 cells and 23% for U87MG cells (Figures 3d and e). Taken together, these results suggest that GBM cells secrete their own Sema3A, which might act as an autocrine signal to promote migration.

Depletion of Sema3A alters cell morphology and inhibits Rac1 activity

To investigate how Sema3A could promote cell migration, we assessed the changes in morphology of Sema3A-depleted cells. We focused on the spreading phenotype of cells and the formation of stress fibers and lamellipodia, actin-rich structures that govern cell migration (Nobes and Hall, 1999). We observed that cells transfected with control siRNA adopt a well-spread phenotype with prominent actin stress fibers indicated by phalloidin staining. In contrast, cells transfected with Sema3A siRNA failed to spread and establish stress fibers and lamellipodia (Figure 4a, i, ii). This effect was quantified by measuring the area of individual cells (Figure 4c). Accordingly, the average total area of control cells was significantly higher than the Sema3A-depleted cells (control siRNA: $2192 \pm 94\,\mu m^2$, $n=135$; Sema3A siRNA: $1186 \pm 42\mu m^2$, $n=160$ $P<0.00001$), showing that Sema3A depletion reduced cell spreading. This phenotype could be recovered when Sema3A-depleted cells were treated with Sema3A/Fc before their attachment (Supplementary Figure 3). Taken together,

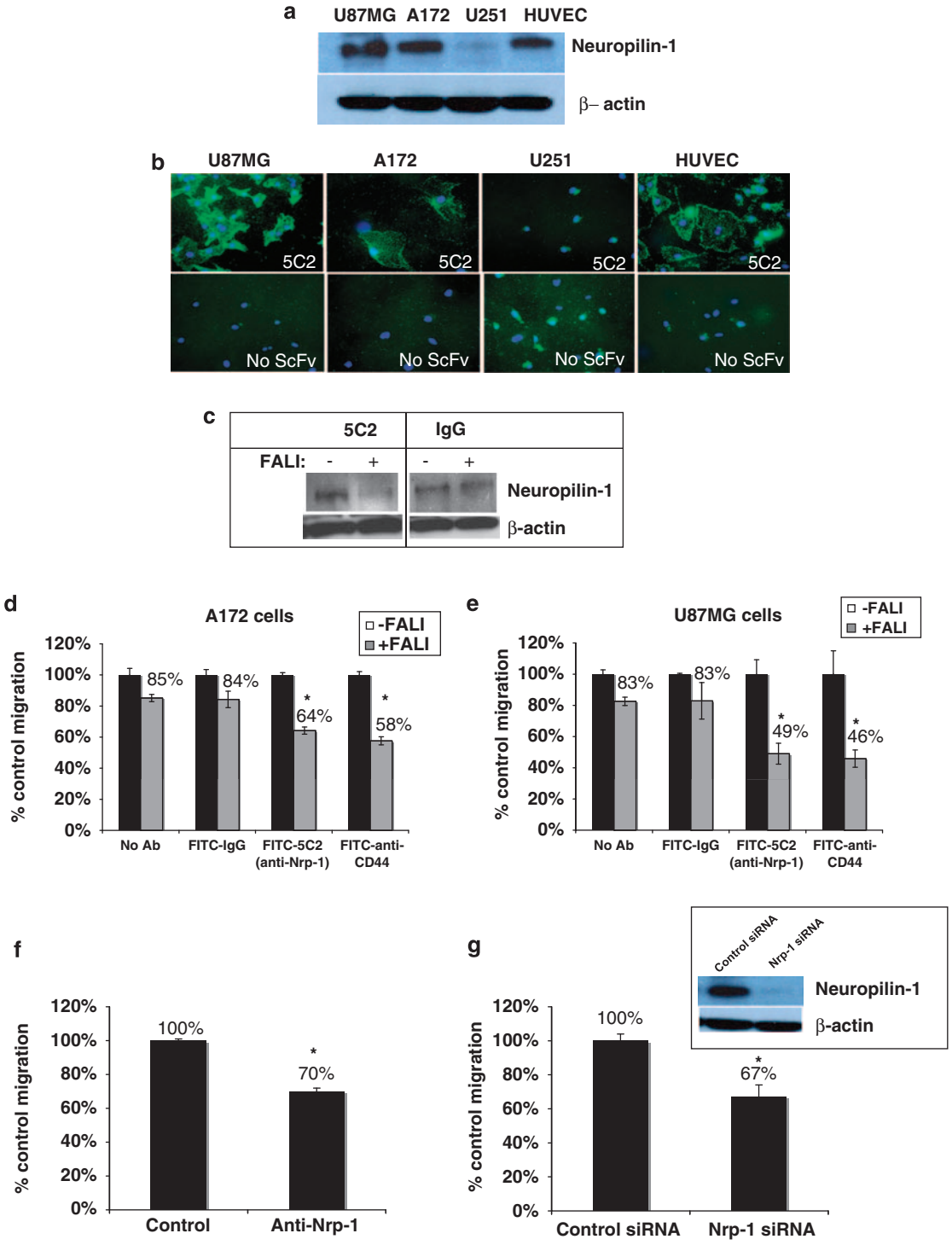


Figure 2 Inhibition of neuropilin-1 decreases glioblastoma multiforme (GBM) cell migration. (**a**) Neuropilin-1 expression in human GBM cell lines A172, U87MG and U251, and human umbilical vein endothelial cell (HUVEC) as assessed by immunoblotting (β-actin is used as control). (**b**) Neuropilin-1 immunofluorescence shows that 5C2 recognizes neuropilin-1 expressing A172, U87MG and HUVEC cells, but not U251 cells. (**c**) Immunoblotting for neuropilin-1 on fluorophore-assisted light inactivation (FALI)-treated cell lysates (β-actin is used as control). FALI with 5C2 causes a reduction in the protein levels of neuropilin-1, an effect not observed with IgG-negative control. (**d**) FALI of neuropilin-1 using fluorescein isothiocyanate (FITC)-5C2 results in inhibition of A172 transwell migration toward a serum chemoattractant (% change in migration with FALI: no Ab: 15%; FITC IgG: 16%; FITC-5C2: 36%; FITC-anti-CD44: 42%). ns indicates nonsignificant; * indicates $P<0.001$, *t*-test. (**e**) FALI of neuropilin-1 using FITC-5C2 results in the inhibition of U87MG transwell migration toward a serum chemoattractant (% change in migration with FALI: no Ab: 17 %; FITC IgG: 17%; FITC-5C2: 51%; FITC-anti-CD44: 54%). ns indicates nonsignificant; * indicates $P<0.001$, *t*-test. (**f**) Function blocking anti-Nrp-1 inhibits A172 migration (% change in migration: 30%). * indicates $P<0.01$, *t*-test. (**g**) Depletion of neuropilin-1 by RNA interference inhibits A172 migration. The inset shows the neuropilin-1 immunoblot on control small interfering RNA (siRNA) or Nrp-1 siRNA (Dharmacon Smartpool, Dharmacon Inc., Lafayette, CO, USA) transfected A172 lysates. % change in migration: 33%. * indicates $P<0.01$, *t*-test.

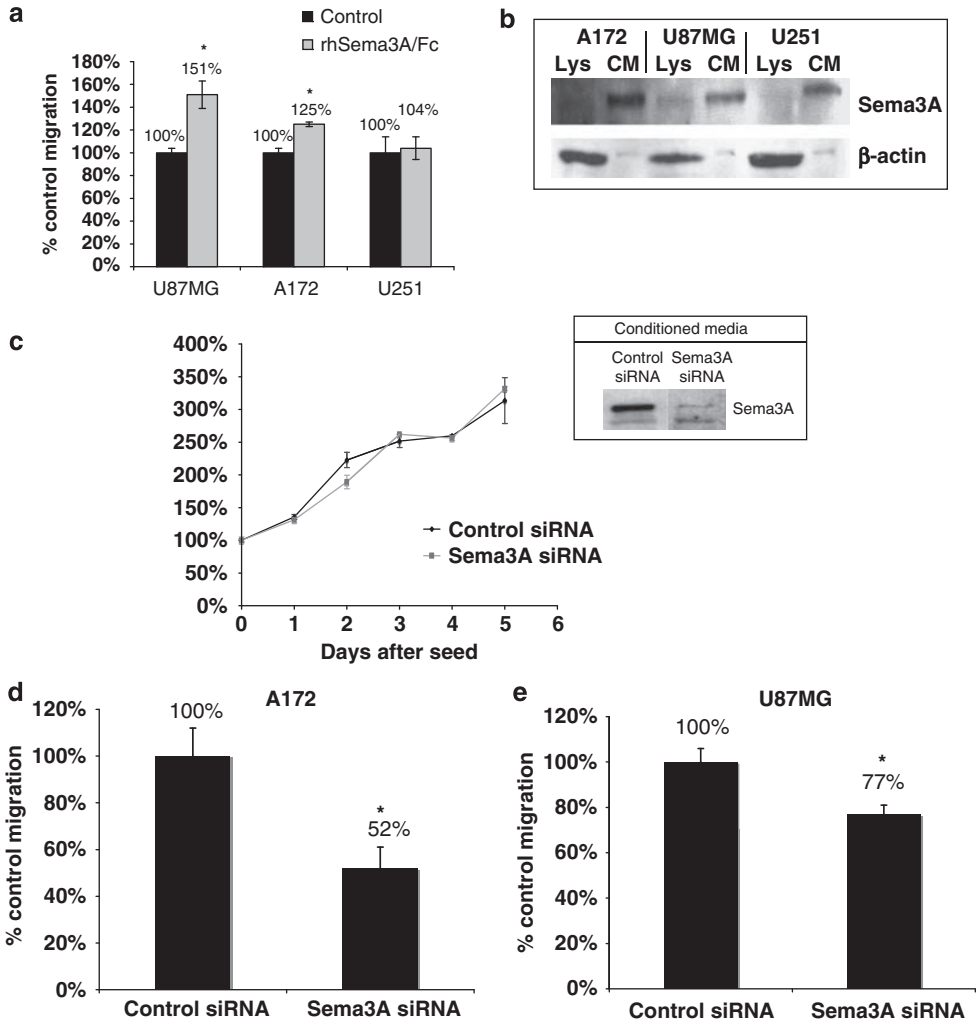


Figure 3 Semaphorin3A (Sema3A) promotes glioblastoma multiforme (GBM) cell migration. (**a**) GBM transwell migration toward a serum chemoattractant significantly increases with Sema3A. The effects of 100 ng/ml rhSema3A/Fc are shown (% change in migration with Sema3A: U87MG: 51 ± 12%; A172: 25 ± 2%; U251: 4 ± 10%). (**b**) Immunoblotting for Sema3A on the lysates (Lys) and conditioned media (CM) collected from U251, A172 and U87MG cells. Sema3A is also found in the CM suggesting that it is secreted from the GBM cells. β-actin is used as a loading control; it is absent from the CM confirming the purity of the soluble CM fraction. (**c**) RNA interference (RNAi)-mediated depletion of Sema3A does not affect cell viability. Sema3A-specific small interfering RNA (siRNA) transfection results in significant reduction of the protein secreted into the CM at 48 h. Growth curves of A172 cells transfected with control (gray lines) or Sema3A siRNA (black lines) are indistinguishable. Shown here is the graph of MTS assay carried out over 5 days. (**d**) RNAi-mediated depletion of Sema3A decreases A172 transwell migration. Cells transfected with Sema3A siRNA migrate significantly less than control siRNA-transfected cells (% difference in migration: 48 ± 11%). * indicates $P<0.01$, *t*-test. (**e**) RNAi-mediated depletion of Sema3A decreases U87MG transwell migration (% difference in migration: 23 ± 4%). * indicates $P<0.01$, *t*-test.

these data suggest that endogenous Sema3A acts in the formation of lamellipodia in GBM cells.

Sema3A facilitates the guidance of developing neurons through repulsion, where the Sema3A binding to its receptors activates the small Rho-GTPase Rac1 (Turner *et al*., 2004). Rac1 is also a mediator of lamellipodia formation in non-neuronal cells, such as fibroblasts (Nobes and Hall, 1999). The observation that Sema3A is involved in regulating the morphology of GBM cells prompted us to check for activation of Rac1 in response to reduction of Sema3A. Using Rac1-GTP pull-down assays on control or Sema3A-depleted cells (Figure 4b), we showed that depletion of Sema3A reduced Rac1 activity compared with control cells (by ~70%), without affecting the total Rac1 levels. These findings indicate that endogenous Sema3A signaling in GBM cells is associated with high Rac1 activity. We then tested whether increasing Rac1 activity would recover the effect of Sema3A depletion. We transfected control or Sema3A-depleted cells with a construct encoding constitutively active Rac1 (CA-Rac1) (Subauste *et al*., 2000). Expression of green fluorescent protein-labeled CA-Rac1 in Sema3A-depleted cells rescued the morphological phenotype (Figure 4a, iii) and increased the cell area to the levels comparable to control cells ($1900 \pm 82 \mu m^2$, $n = 192$) (Figure 4c). Taken together, our results suggest that Sema3A signaling in GBM cells involves the activation of Rac1, providing a potential mechanism to promote GBM cell migration.

Depletion of Sema3A inhibits GBM dispersal

To mimic the three-dimensional dispersal of GBMs, we formed spheroids of GBM cells by the hanging drop method (Del Duca *et al*., 2004) and used them in a collagen invasion assay. We transfected A172 and U87MG cells with control or Sema3A siRNA and formed spheroids of similar size. At 24 h after implantation, the control spheroids dispersed readily, whereas the Sema3A-depleted spheroids dispersed significantly less (Figures 5a and b). The reduction in dispersal could be recovered by exogenous addition of Sema3A/Fc. For A172 spheroids, the number of dispersed control cells was 68 ± 6, compared with 18.5 ± 3.5 Sema3A-depleted cells ($P<0.05$, *t*-test). The Sema3A/Fc addition significantly recovered the decrease in dispersal (87.5 ± 11.5 ($P<0.05$, *t*-test)) (Figure 5c). Similar results were observed for U87MG spheroids; the number of control cells and Sema3A-depleted cells were 236 ± 28 and 119 ± 14, respectively ($P<0.05$, *t*-test). Dispersal of Sema3A-depleted spheroids increased significantly with Sema3A/Fc treatment (177 ± 9 ($P<0.05$, *t*-test)) (Figure 5d). Taken together, these results implicate Sema3A as a promoter of GBM dispersal.

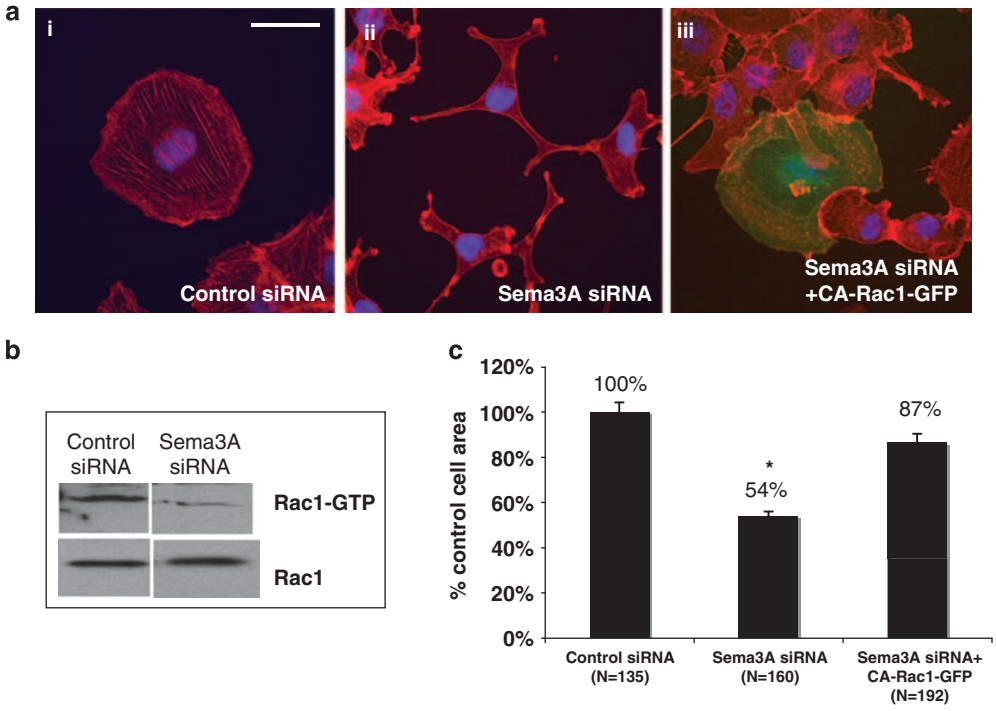
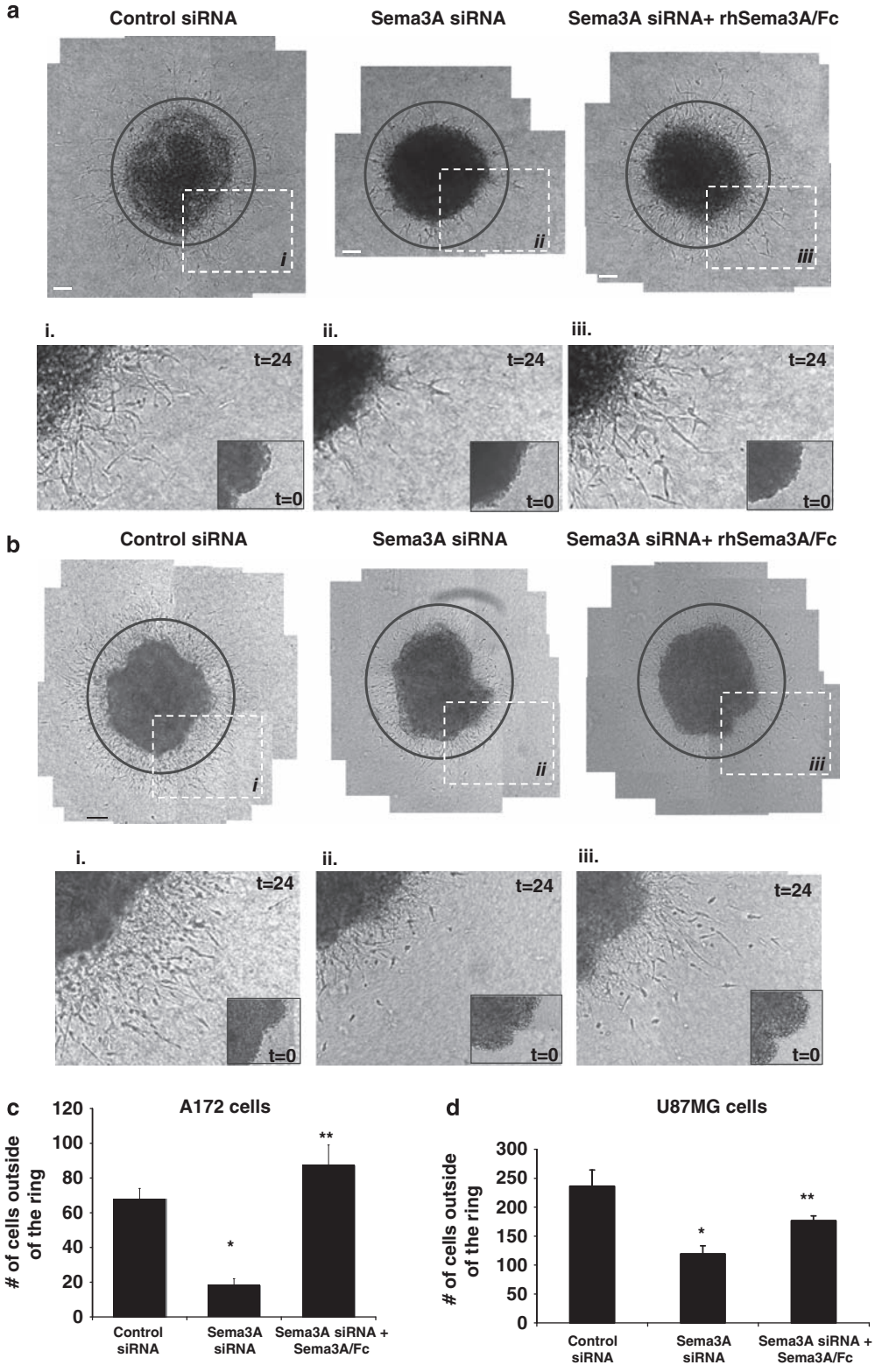


Figure 4 Depletion of semaphorin3A (Sema3A) alters cell morphology and inhibits Rac1 activity. (**a**) F-actin staining of control small interfering RNA (siRNA) (**i**), Sema3A siRNA (**ii**) or Sema3A siRNA + constitutively active Rac1 (CA-Rac1)-green fluorescent protein (GFP) (**iii**) transfected A172 cells, which were seeded on laminin-coated slides. Cells that adhered to laminin were stained with phalloidin (red) for actin cytoskeleton, or DAPI (blue) for nuclei. The green fluorescence is indicative of the GFP signal. The control cells adopt a well-spread phenotype, whereas Sema3A-depleted cells fail to spread on laminin. CA-Rac1-GFP recovers the effect of Sema3A depletion on cell morphology. Scale bars: 50 μm. (**b**) Effects of Sema3A depletion on Rac1 activity. GTP-bound activated Rac1 was immunoprecipitated from A172 cell lysates transfected with control or Sema3A siRNA and immunoblotted with anti-Rac1. The active Rac1 levels markedly decrease, whereas the total Rac1 levels remain unchanged with Sema3A siRNA. (**c**) Effects of Sema3A depletion on cellular morphology. Area of cells stained with phalloidin was measured using OpenLab tools and presented as percent cell area. Sema3A siRNA transfected cells exhibit a significant decrease in average cell area compared with control cells (difference in area: 46%, $P<0.0001$, *t*-test). Transfection of CA-Rac1-GFP into Sema3A-depleted cells significantly increases the cell area ($P<0.0001$, *t*-test).

Figure 5 Depletion of semaphorin3A (Sema3A) decreases glioblastoma multiforme cell dispersal. (**a**) Representative composite DIC images of A172 spheroids that have been implanted into collagen and dispersed for 24 h. The bottom panels (**i**–**iii**) show magnified images of outlined fields of dispersed cells as well as their corresponding time = 0 images. The extent of cell dispersal is significantly less in Sema3A small interfering RNA (siRNA) spheroids compared with controls. The reduction in dispersal by Sema3A depletion can be recovered by adding back exogenous Sema3A. Scale bar: 100 μm. (**b**) Representative composite DIC images of U87MG spheroids that have been implanted into collagen and dispersed for 24 h. The bottom panels show magnified images of cells dispersing. Scale bar: 100 μm. (**c**) Quantification of dispersal from A172 spheroids. The number of cells that have reached more than or equal to half a radius away from the spheroid surface are counted and plotted. Control siRNA: 68 ± 6; Sema3A siRNA: 18.5 ± 3.5; Sema3A siRNA + rhSema3A/Fc: 87.5 ± 11.5. * denotes comparison of control vs Sema3A siRNA spheroids; ** denotes comparison of Sema3A siRNA vs Sema3A siRNA + rhSema3A/Fc spheroids. *, ** indicate $P<0.05$, *t*-test. (**d**) Quantification of dispersal from U87MG spheroids. The number of dispersed cells are counted and plotted as an average of eight spheroids/group. Control siRNA: 236 ± 28; Sema3A siRNA: 119 ± 14; Sema3A siRNA + rhSema3A/Fc: 177 ± 9. * denotes comparison of control vs Sema3A siRNA spheroids; ** denotes comparison of Sema3A siRNA vs Sema3A siRNA + rhSema3A/Fc spheroids. *, ** indicate $P<0.05$, *t*-test.

Extracellular Sema3A decreases GBM cell adhesion in a neuropilin-1-dependent manner

The mode of Sema3A action on motility has previously been associated with the regulation of adhesion. For example, during vascular morphogenesis, exogenously applied Sema3A can inhibit the substrate adhesion of endothelial cells (Serini *et al*., 2003). To mimic the presence of secreted Sema3A in the vicinity of GBM cells, we presented Sema3A as part of the extracellular matrix (ECM) and observed the cell-substrate adhesion



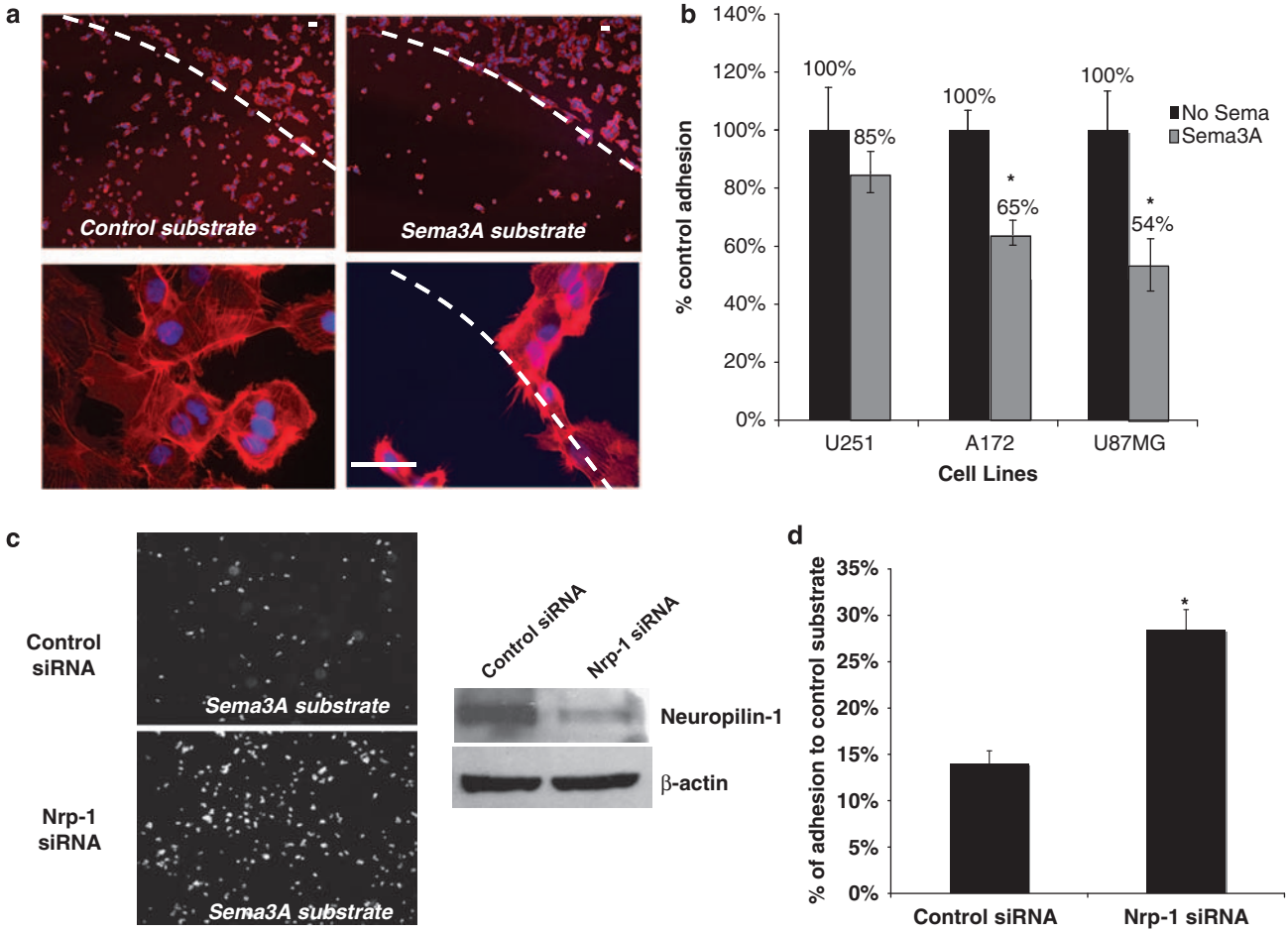


Figure 6 Extracellular semaphorin3A (Sema3A) blocks adhesion in a neuropilin-1-dependent manner. (**a**) Representative images of A172 cells adhering to control vs Sema3A-rich laminin substrates and their surrounding laminin beds. Upper panels: low-magnification (× 5) images of cells stained with phalloidin show that the number of cells on Sema3A-rich substrate is significantly lower than that on control substrate. Lower panels: high-magnification (× 40) images of cells at the borders of Sema3A and control substrates. The cells that are localized at the border of Sema3A substrate undergo local cytoskeletal collapse as shown by the depolymerizing actin cables. Shown in blue are nuclei stained with DAPI. Scale bars: 50 μm. (**b**) Quantification of adhesion in response to Sema3A (5 μg/ml) among three glioblastoma multiforme cell lines. The graph shows the number of cells that adhered to Sema3A substrate compared with control substrate (% change in adhesion: U251 cells: 15 ± 9%; A172 cells 35 ± 7%; U87MG cells 46 ± 4%). * indicates $P<0.05$, *t*-test. (**c**) Depletion of neuropilin-1 by RNA interference (RNAi) partially abolishes the response to Sema3A. Left: A172 cells transfected with control or neuropilin-1 (Nrp-1)-specific small interfering RNA (siRNA) are subjected to the adhesion assay described in panel a. The decreased adhesion response to Sema3A (10 μg/ml) is partially abolished by the depletion of neuropilin-1 by RNAi. Right: immunoblotting for neuropilin-1 on A172 cell lysates transfected with control or Nrp-1 siRNA shows that there is a significant decrease in neuropilin-1 protein. (**d**) Quantification of adhesion to Sema3A substrate (control siRNA cells: 14 ± 1%, Nrp-1 siRNA cells: 28 ± 2%, $P<0.0005$, *t*-test).

behavior. Sema3A-rich ECM was generated by mixing Sema3A and laminin, and spotting this mixture in a bed of laminin of equal concentration. We also formed control spots in which the volume of Sema3A was substituted with control buffer.

As shown in Figure 6a, A172 cells adhered equally well to both the control spots and the surrounding laminin bed. In contrast, the cells avoided Sema3A-rich spots, but preferentially adhered to surrounding laminin devoid of Sema3A. Phalloidin staining showed that the actin cytoskeleton was intact in cells that adhered to control spots. In contrast, the few cells that did adhere to Sema3A-rich spots adopted a collapsed phenotype (data not shown). Interestingly, cells that adhered adjacent to the Sema3A-rich spots underwent local cytoskeletal collapse at the Sema3A interface. These observations suggest that GBM cells undergo local regulation of cytoskeletal collapse and de-adhesion when they are exposed to an external Sema3A signal.

To quantify the inhibition of cell adhesion, we counted the number of cells inside the control and Sema3A-rich spots and observed a 35% inhibition of adhesion with Sema3A (Figure 6b). The reduction in adhesion was dose-dependent with a larger inhibition with higher Sema3A concentrations in the spots (data not shown). In the same assay, U87MG cells significantly responded to Sema3A by 46% decreased adhesion, U251 cells remained minimally affected (Figure 6b). The observed

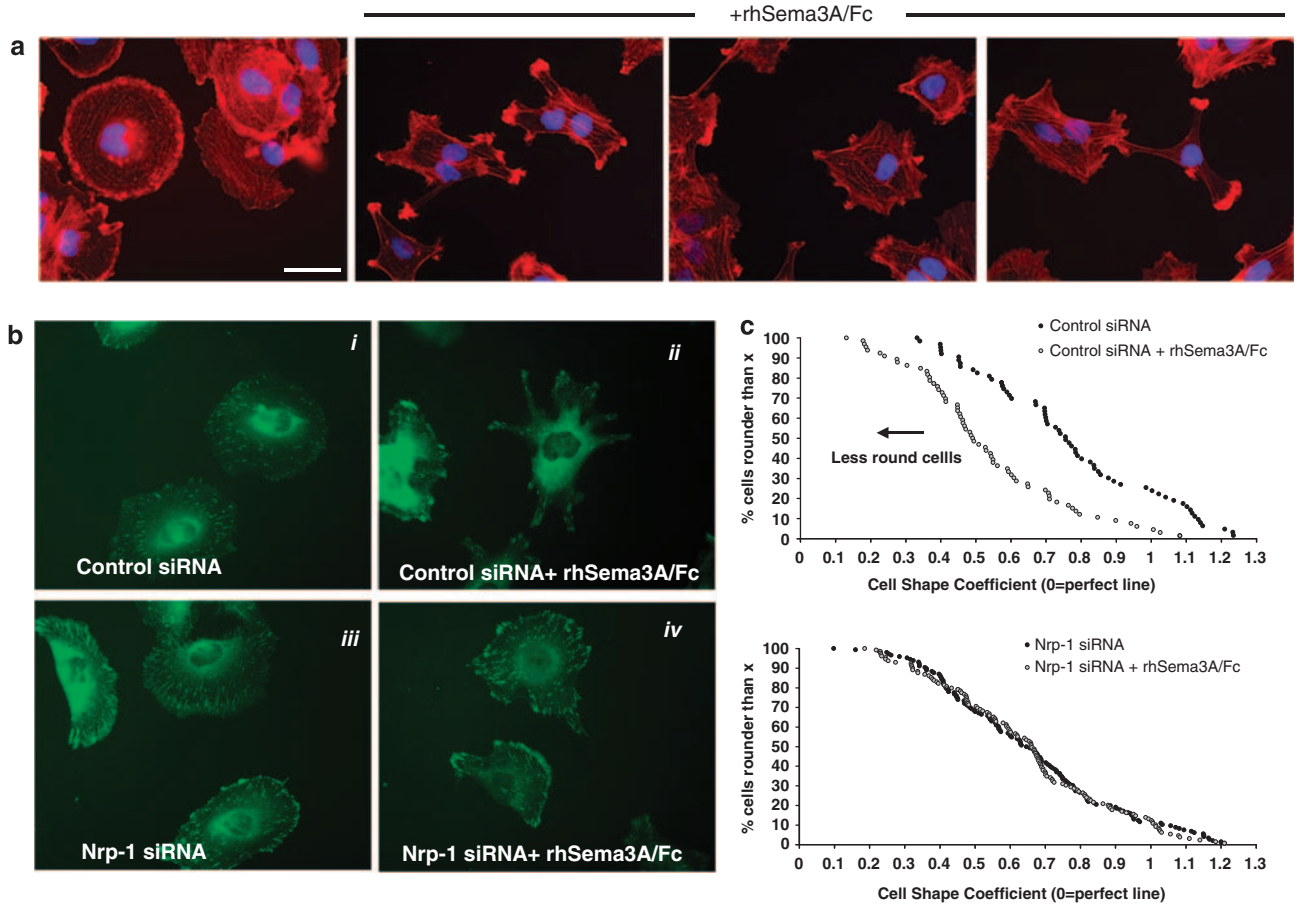


Figure 7 Semaphorin3A (Sema3A) decreases focal adhesions in a neuropilin-1-dependent manner. (**a**) Sema3A treatment causes collapse. Cells, first seeded on laminin for 1 h, were treated with exogenous rhSema3A/Fc for 30 min. Phalloidin staining shows that cells are collapsing in response to Sema3A. Note the still intact stress fibers in these cells. (**b**) Representative images of cell stained for Vinculin (green). (**i**) Control small interfering RNA (siRNA)-transfected cells treated with control buffer; (**ii**) Control siRNA-transfected cells treated with rhSema3A/Fc; (**iii**) Nrp-1 siRNA-transfected cells treated with control buffer; (**iv**) Nrp-1 siRNA-transfected cells treated with rhSema3A/Fc. (**c**) Chang plots showing cell shape measurements. Cell shape coefficient is defined as a function of cell area/(cell perimeter)^2, where 0 denotes a perfect line. Top: control cells become more elongated/less circular after rhSema3A/Fc treatment (shape coefficient average ± s.e.m. control siRNA cells ($n=62$): 0.77 ± 0.03; control siRNA + rhSema3A/Fc cells ($n=66$): 0.52 ± 0.03, $P<0.0001$, *t*-test). Bottom: Nrp-1 siRNA-transfected cells do not respond to rhSema3A/Fc and remain circular (shape coefficient average ± s.e.m.: Nrp-1 siRNA cells ($n=146$): 0.66 ± 0.02; Nrp-1 siRNA + rhSema3A/Fc cells ($n=129$): 0.65 ± 0.02, $P=0.79$, *t*-test).

correlation between the magnitudes of Sema3A responses with the endogenous neuropilin-1 levels in the three GBM cell lines suggested that the effect of Sema3A on GBM cell adhesion is neuropilin-1 dependent. To test this notion further, we depleted neuropilin-1 using RNAi in A172 cells. Depletion of neuropilin-1 partially abolished the response to Sema3A, indicating that this behavior is neuropilin-1 dependent (Figures 6c and d).

To test the effect of exogenously applied Sema3A on cell adhesion, we uniformly presented Sema3A in the cell media. In the absence of Sema3A, cells remained well spread with prominent stress fibers. Sema3A treatment caused changes in the actin cytoskeleton resembling a collapse event and abrogated the circular shape of cells toward a more elongated/less circular form (Figure 7a). To further assess the effects of exogenously applied Sema3A on cells, we examined focal adhesions using immunofluorescence for Vinculin (Ziegler *et al*., 2006). In the absence of Sema3A, cells contained Vinculin-positive focal adhesions (Figure 7b, i). Sema3A treatment caused rearrangement of these adhesions in accordance with a more elongated/less circular phenotype (Figure 7b, ii). The effect of exogenously applied Sema3A was dependent on neuropilin-1, as the Nrp-1 siRNA transfected cells remained unaffected by Sema3A (Figure 7b, iii, iv). To quantify the changes in the cell shape, we graphed the cell shape coefficient of individual cells using Chang plots. The shape of control cells shifted toward a less circular state with rhSema3A treatment, whereas Nrp-1-depleted cells remained unchanged (Figure 7c). Taken together, these data suggest a possible mechanism in which exogenous Sema3A facilitates GBM dispersal by promoting local de-adhesion of cells from the extracellular environment in a neuropilin-1-dependent manner, permitting cells to migrate away from the tumor.

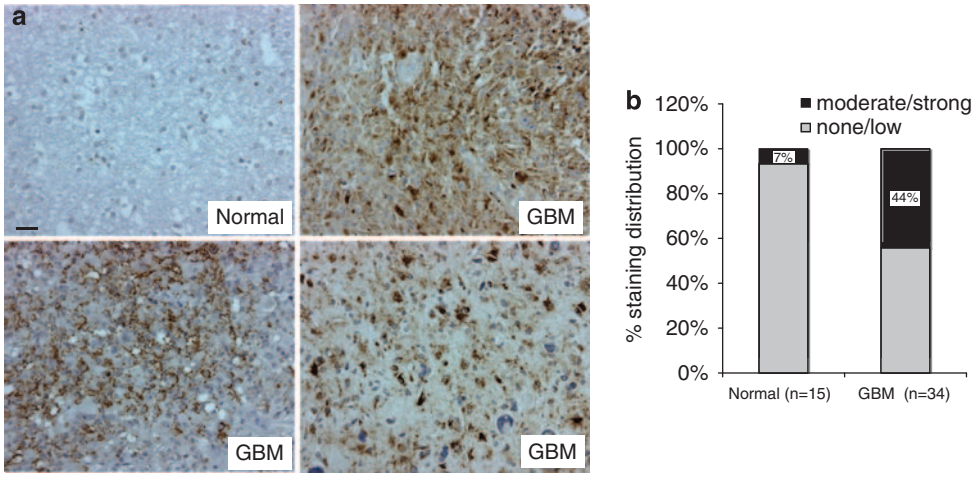


Figure 8 Semaphorin3A (Sema3A) expression in human glioblastoma multiformes (GBMs). (**a**) Immunohistochemical analysis of Sema3A in human GBM tissue. Sema3A is absent from the normal white matter, but highly expressed in GBM. The staining is cytoplasmic and extracellular as depicted by the localized intracellular and the punctate staining patterns. Scale bar: 100 μm. (**b**) Quantification of Sema3A immunohistochemistry carried out on a tissue microarray ($n=34$ GBM, $n=15$ normal brain). In all, 44% of GBMs have elevated Sema3A staining and this is significantly higher than the ratio observed for normal brain ($P<0.0001$, χ^2-test).

Sema3A is highly expressed in human GBMs compared with normal brain

To test the clinical relevance of our findings, we examined human GBM samples for their Sema3A expression by immunohistochemistry on GBM tissue microarrays. We assessed 34 GBM and 15 non-neoplastic brain samples and observed that Sema3A is expressed by a subset of GBM tumors but not by the white matter of the non-neoplastic brain (Figure 8a). The pattern of expression was reminiscent of a soluble protein, as evidenced by the punctate cytoplasmic/extracellular staining in GBM samples, in agreement with the reports of Sema3A expression in embryonic neurons (De Wit *et al*., 2005). The expression was predominantly in neoplastic astrocytes as identified by their nuclear atypia. To make a quantitative assessment of staining, we ranked the staining intensity as none, low, moderate and strong. The analysis of staining patterns in all sections showed a significant overexpression of Sema3A compared with normal brain; 44% (15/34) of GBMs were moderately or strongly positive for Sema3A compared with only 7% (1/15) of the non-neoplastic brain samples (χ^2-test, $P<0.0001$) (Figure 8b). Our observation of Sema3A overexpression in human GBMs indicates the clinical relevance of our *in vitro* data and suggests that Sema3A and neuropilin-1 could act as a novel autocrine ligand–receptor pair to enhance glioblastoma cell infiltration into the brain.

Discussion

In this study, we identified neuropilin-1 through a functional proteomic screen for proteins involved in cancer cell invasion. We showed that neuropilin-1 in GBM cells might act as a receptor for endogenously expressed Sema3A to increase migration and dispersal *in vitro*. Exogenous Sema3A blocks adhesion of GBM cells to the ECM in a neuropilin-1-dependent manner. Taken together, our results suggest that Sema3A signaling causes GBM cell de-adhesion permitting dispersal away from the tumor mass. The overexpression of Sema3A in human GBMs compared with the non-neoplastic brain attests to the clinical relevance of our findings.

When a tumor cell encounters a migration-promoting stimulus, it polarizes and extends lamellipodia and filopodia in the direction of migration (Ridley *et al*., 2003), which are enriched in signaling proteins that allow the cell to move forward through interactions with the underlying cytoskeleton and ECM. To facilitate forward movement, the cells modify their contact with ECM and breakdown their adhesions at their trailing edge. In this study, we have established that Sema3A is an important neuropilin-1 ligand required for GBM cell migration and dispersal, and shown that exogenous Sema3A blocks the adhesion of GBM cells to the ECM. This suggests that Sema3A signaling within GBM microenvironment can ease migration away from the tumor by facilitating the de-adhesion step of the migration cycle. It will be of high interest to test whether Sema3A can regulate the polarity of cells by also modulating other receptors, such as integrins in GBM cells. In addition, the neuropilin-1-dependent action of Sema3A prompts further studies on assessing the distribution and bioavailability of neuropilin-1 within different compartments of GBM cell surface (for example, leading edge vs trailing edge).

Accumulating evidence suggests that neuropilin-1 is overexpressed in several cancers and its high expression is correlated with poor prognosis (Ding *et al*., 2000; Kawakami *et al*., 2002; Roche *et al*., 2002; Vanveldhuizen *et al*., 2003; Broholm and Laursen, 2004; Fukahi *et al*., 2004; Li *et al*., 2004; Osada *et al*., 2004; Parikh *et al*., 2004; Wey *et al*., 2005; Ellis, 2006; Kreuter

et al., 2006; Hu *et al*., 2007; Staton *et al*., 2007; Vales *et al*., 2007). In addition to its role as a Sema3A receptor, neuropilin-1 is a co-receptor for a specific isoform of VEGF165 and guides endothelial cell migration during vascular morphogenesis (Soker *et al*., 1998; Lee *et al*., 2002). The role of neuropilin-1 in tumors has been mostly studied in the context of its interaction with VEGF165 and related to tumor angiogenesis and tumor cell survival (Miao *et al*., 2000; Bachelder *et al*., 2001). A recent study suggested that neuropilin-1 contributes to tumor progression by potentiating the activity of hepatocyte growth factor as well (Hu *et al*., 2007). However, the role of neuropilin-1 in tumor progression has not been studied in the context of its Sema3A interactions. Although little is known about how Sema3A functions in human cancers, including GBM, previous studies comprehensively defined a role for Sema3A in the nervous system as an axon guidance molecule.

In the nervous system, neuropilin-1 acts as a receptor for Sema3A and guides the directional motility of axonal growth cones (Kolodkin *et al*., 1997; Turner *et al*., 2004; Tran *et al*., 2007). Sema3A signaling requires the formation of neuropilin-1 and Plexin-A receptor complexes at the cell surface. The binding of Sema3A triggers the intracellular signaling that modulates small GTPases. Overall, Sema3A induced activation of Rac1 together with inactivation of R-Ras inhibits integrin-mediated adhesion and results in repulsion (Halloran and Wolman, 2006). Our results with cell morphology analysis imply that Rac1 is an essential player downstream of Sema3A in GBM cells. This finding is important for two reasons. First, it suggests that GBM cells might be equipped with the machinery for Sema3A signaling similar to neurons. Second, it links GBM cell-expressed Sema3A with a known inducer of GBM cell motility, Rac1. In fact, the role of Rac1 in GBM has been suggested before and independent of its link with Sema3A (Nakada *et al*., 2006). It will be of great interest to address the expression and function of other Sema3A signaling components in GBMs. Our PCR analyses (Supplementary Figure 4) as well as a study by others (Raper, 2000) showed the expression of PlexinA receptors in human GBM cell lines. Therefore, functional studies with Plexins in GBM cells will further our understanding of Sema3A/neuropilin-1-mediated GBM dispersal.

Although Sema3A is mainly known as an axonal repellent, it has also been shown to act as an attractant for developing dendrites and radial migration of cortical neurons (Polleux *et al*., 2000; Chen *et al*., 2008). During cortical neuron migration, Sema3A is regionally expressed and attractive for neuropilin-1-expressing neurons. RNAi-mediated neuronal depletion of neuropilin-1 blocks proper lamination by blocking the Sema3A signaling. Similarly, our findings suggest a pro-migratory function for Sema3A/neuropilin-1 signaling in GBMs. Therefore, there could be a shared mechanism between GBM dispersal and radial migration of cortical neurons. In other studies, the bifunctional nature of Sema3A in the nervous system has been attributed to the differential availability of intracellular signaling effectors, such as cyclic nucleotides (cGMP) (Zhou *et al*., 2008). It was shown that elevating intracellular cGMP levels could switch a neuronal Sema3A response from repulsive to attractive (Song *et al*., 1998). In fact, differential expression of guanylate cyclase, which elevates cGMP levels, in different compartments of developing neurons determines the responses to Sema3A. The pro-migratory effect of Sema3A on GBM cells could possibly be linked to the intracellular availability and localization of cGMP as well. Other factors on the cell surface, such as L1-CAM, could modulate Sema3A effects and shift neuronal migration response from repulsion to attraction (Castellani *et al*., 2000, 2002). Therefore, functional studies addressing the role of L1-CAM and cGMP in Sema3A-mediated GBM cell migration will be crucial to further understand Sema3A signaling in GBMs.

The loss of adhesion in response to exogenously applied Sema3A suggests that Sema3A released by tumor cells and deposited in the ECM can act in paracrine to modulate adhesion. At first glance, this behavior seems at odds with how Sema3A acts during growth cone motility. During axon guidance, growth cones undergo collapse when exposed to exogenous Sema3A when tension exceeds adhesion of the growth cone's leading edge to ECM (Halloran and Wolman, 2006). Our findings suggest that the role of Sema3A in GBM migration/dispersal is complex and perhaps biphasic. There is a biphasic relationship between the adhesive strength and cell motility of cells such that there is optimal adhesion for maximal cell motility (DiMilla *et al*., 1993; Cox and Huttenlocher, 1998). Similarly, our findings are consistent with an optimal Sema3A concentration for GBM dispersal such that GBM cells are able to release their adhesions efficiently and move away from one another. We would predict that more than optimal expression of Sema3A in GBM cells might also tilt the balance of adhesion towards less dispersal. In fact, our preliminary studies show that forced overexpression of Sema3A in GBM cells causes decreased dispersal as opposed to an increase. Taken together, we suggest a 'biphasic model' for the role of Sema3A in dispersal in which the concentration (and perhaps direction) of Sema3A exposure determines the effects on GBM dispersal (Supplementary Figure 5).

We find Sema3A is expressed in a subset of human GBMs compared with the non-neoplastic brain. This observed heterogeneity in expression could potentially establish Sema3A signaling in both autocrine (on the tumor cells secreting Sema3A) as well as paracrine manner (by setting up gradients in the microenvironment and modulating cell-substrate adhesion). Although the expression of semaphorins has previously been reported in glioma cell lines (Rieger *et al*., 2003), this is the first study that examines the pattern of Sema3A expression in GBM tissues. In studies using cell lines, Sema3A has been suggested to be either pro-invasive or anti-invasive. A study with breast cancer cell lines showed that Sema3A knockdown increased cell migra-

tion perhaps because these cells were primarily responsive to VEGF165 (unlike the GBM cells that we examined) and that Sema3A could compete with VEGF165 (Bachelder *et al*., 2003). Our findings concur with a recent study that implicated Sema3A as a pro-invasive protein in prostate cancer cells, which had enhanced migration when presented with Sema3A exogenously (Muller *et al*., 2007). Therefore, the role of Sema3A in cancer cell invasion may depend on the context of other expressed factors as well as how the tumor cells receive the signal.

The expression of Sema3A along with its receptor neuropilin-1 in GBM cells raises the possibility that this ligand–receptor pair could serve as an autocrine signaling mechanism. Recently, several anti-neuropilin-1 agents that blocked VEGF binding were tested in animal models (Pan *et al*., 2007). Our findings on GBM cells suggest an additional role for neuropilin-1, in which it acts as a Sema3A receptor and facilitates tumor cell dispersal. As tumor cell dispersal is a critical aspect of GBMs, limiting this behavior would likely improve the outcome of tumor resection. This raises the possibility that targeting multiple signaling pathways mediated by neuropilin-1, including Sema3A, could serve as a means of improving GBM prognosis.

Materials and methods

Cell culture

HT1080 human fibrosarcoma and A172, U87MG and U251 glioblastoma cell lines were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in humidified incubators with 7% CO_2. Human umbilical vein endothelial cells were cultured in Medium 200 (Cascade Biologics, Portland, OR, USA) supplemented with low-serum growth supplement (Cascade Biologics).

Fluorophore-assisted light inactivation

Antibodies used for FALI were as follows: anti-β1-integrin (JB1, Chemicon, Temecula, CA, USA), anti-Nrp-1 (recombinant ScFv 5C2), anti-CD44 (MAB4065F, Chemicon) and mouse IgG (Pierce, Milwaukee, WI, USA). All antibodies were conjugated to FITC as previously described (Eustace *et al*., 2004). Cells were collected with Versene (Invitrogen) in phenol red-free HBSS (Invitrogen) and incubated with the antibodies for 1 h with gentle rocking at room temperature. The cells were then transferred to 96-well plates in triplicates and irradiated with blue-filtered light for 1 h on ice using a slide projector (Ektagraphic III, Kodak, New Haven, CT, USA). A control plate was kept on ice without irradiation. Cells that were treated with FALI were immediately used for migration/invasion, or harvested after 1 h for immunoblotting.

Adhesion assay

To present Sema3A as part of the ECM, nitrocellulose-coated coverslips were spotted with mixtures of 10 μg/ml laminin (Invitrogen) that contained 5–10 μg/ml rhSema3A/Fc (R&D Systems, Minneapolis, MN, USA) or control buffer (0.1% bovine serum albumin (BSA)). The remaining regions were filled with an equal concentration of laminin and blocked with 2.5% BSA. Cells were collected with Versene and incubated on these coverslips at 37 °C for 90 min. After rinsing with PBS, the attached cells were fixed, visualized by fluorescence microscopy (Zeiss-Axioplan, Carl Zeiss Microimaging Inc, Thornwood, NY, USA) and counted using OpenLab (Improvision Inc., Waltham, MA, USA). To present Sema3A as a part of the media, cells were first attached to laminin-coated slides for 90 min, treated with 5 μg/ml rhSema3A/Fc for 30 min and then fixed to analyse focal adhesions by immunofluorescence.

Other methods

Transfections, immunohistochemical and quantitative reverse transcriptase–PCR analysis of GBM specimens, immunoblotting, immunofluorescence, cell morphology analysis, cell proliferation, cell migration, spheroid dispersal, high-throughput screening and Rac1 activation assays are described in the Supplementary information.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)