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SPARC-like 1 Regulates the Terminal Phase of Radial Glia-Guided Migration in the Cerebral Cortex

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Summary

Differential adhesion between migrating neurons and transient radial glial fibers enables the deployment of neurons into appropriate layers in the developing cerebral cortex. The identity of radial glial signals that regulate the termination of migration remains unclear. Here, we identified a radial glial surface antigen, SPARC (secreted protein acidic and rich in cysteine)-like 1, distributed predominantly in radial glial fibers passing through the upper strata of the cortical plate (CP) where neurons end their migration. Neuronal migration and adhesion assays indicate that SPARC-like 1 functions to terminate neuronal migration by reducing the adhesivity of neurons at the top of the CP. Cortical neurons fail to achieve appropriate positions in the absence of SPARC-like 1 function in vivo. Together, these data suggest that antiadhesive signaling via SPARC-like 1 on radial glial cell surfaces may enable neurons to recognize the end of migration in the developing cerebral cortex.

Introduction

Neurons in the cerebral cortex are generated in a precise inside-out sequence of genesis (Angevine and Sidman, 1961; Rakic, 1974). The acquisition of their final position during development is achieved through a process of active migration of postmitotic neurons from the proliferative ventricular (VZ) and subventricular (SVZ) zones to their final sites of differentiation in the cortical plate. Early generated neurons of the preplate and deeper layers migrate radially using somal translocation, whereas later generated neurons utilize elongated radial glial guides to attain their more distant locations at the interface between cortical plate (CP) and marginal zone (MZ) (Sidman and Rakic, 1973; Rakic, 1972, 2003; Miyata et al., 2001; Noctor et al., 2001; Nadarajah et al., 2001; Hatten and Mason, 1990; Takahashi et al., 1990). Cortical interneurons migrate tangentially into the cerebral wall from the lateral ganglionic eminence prior to radial glial-guided migration to the cortical plate (Anderson et al., 1997; Ang et al., 2003; de Carlos et al., 1996).

Irrespective of the type or origin, neurons must migrate to the same address at the CP/MZ interface. At this interface, the movement of neurons stops abruptly and cohorts of neurons begin to assemble into their respective layers. This final stage of neuronal migration is the least explored aspect of neuronal migration, in spite of its significance for genetic and acquired cortical malformations (Rakic, 1988; Rakic and Caviness, 1995; Olson and Walsh, 2002). The signal to terminate neuronal cell migration is thought to be provided either by the afferent fibers that migrating neurons encounter near their target location or by the ambient neuronal cell population that had already reached their final position (D'Arcangelo et al., 1995; Hatten and Mason, 1990; Ogawa et al., 1995; Sidman and Rakic, 1973). Alternatively, a change in the cell surface properties of the radial glial substrate at the top of the cortical plate may signal a migrating neuron to stop, detach, and differentiate. To examine this hypothesis, we initiated studies to identify and characterize cell surface cues specific to radial glial cells that may be engaged in selective cell recognition, adhesion, or translocation events necessary to signal neurons to terminate their migration.

We identified a radial glial surface based molecule, SPARC-like 1 (SC1), that is primarily localized in the segment of radial glia spanning the upper cortical plate, where neurons terminate their migration. Inhibition of SPARC-like 1 in vivo perturbs the appropriate laminar placement of cortical neurons. BrdU birthdating indicates neuronal misplacement in SC1 mutant mice. In vitro, SPARC-like 1 inhibits embryonic cortical neuron-glial adhesion. Ectopic expression of SPARC-like 1 in vivo inhibits normal neuronal migration. The temporal and spatial distribution of SPRC-like 1 during cortical development in regions where neurons terminate their migration and its antiadhesive activity, both in vitro and in vivo, suggest that it may function as a cue for cessation of neuronal migration by enabling neuronal detachment from radial glial guides at their final destination in the developing cerebral cortex.

Results

Developmental Distribution of a Radial Glial-Based Terminator of Neuronal Migration

To identify molecules that are present on the radial glial cell surface that can cause neurons to end their migration and begin layer formation, a panel of 97 radial glia-immunoreactive monoclonal antibodies (mAbs), generated against whole-cell extracts of embryonic spinal cord and brain tissues using the cyclophosphamide immunosuppression paradigm (Anton et al., 1994), was screened in the developing rat cerebral wall. One of the antibodies from this panel, RGRS1 (Radial Glial Release

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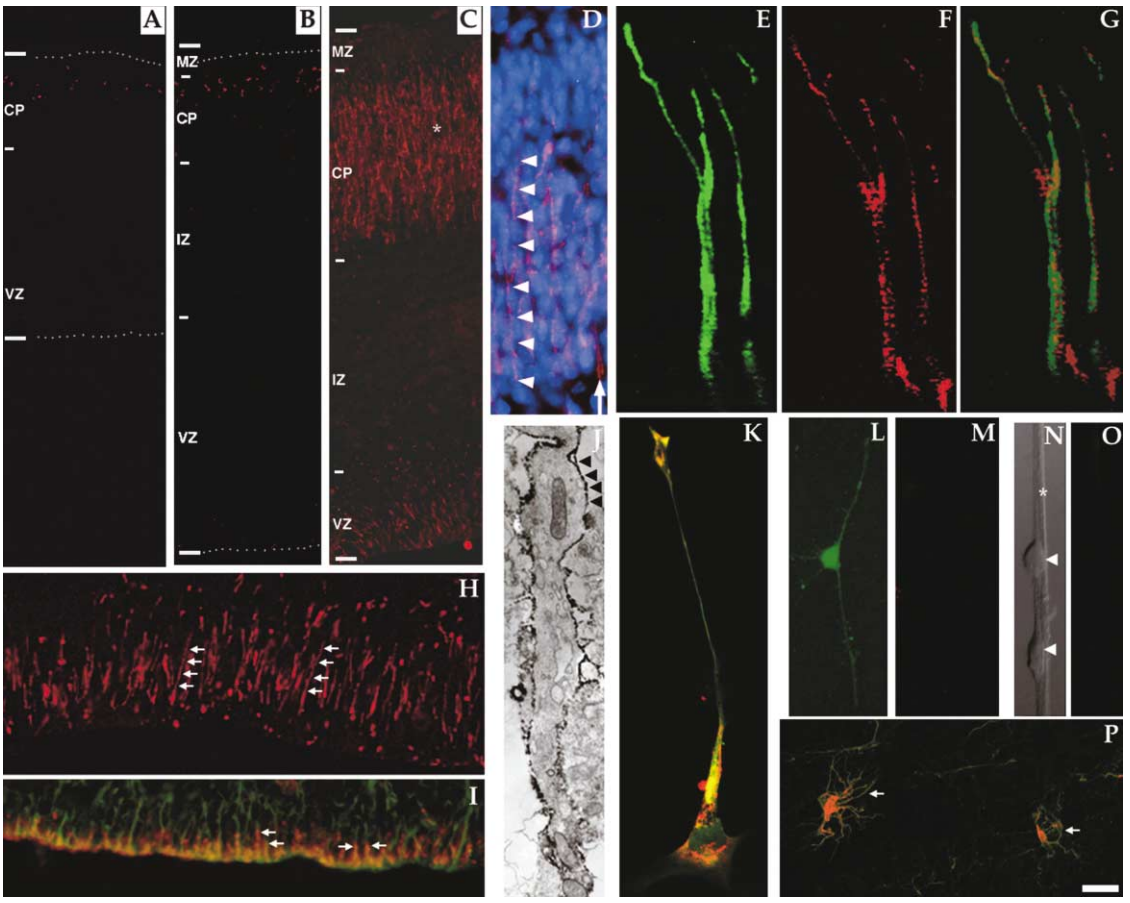

Figure 1. Distribution of RGRS1 in Developing Cerebral Cortex

(A and B) RGRS1 is distributed punctately at the top of the CP at E13.5 (A) and E16 (B).

(C) By E18, immunoreactivity of radially oriented processes (asterisk) is noticeable at the cortical plate and ventricular zone. Immunoreactivity is absent in the intermediate zone, which contains migrating neurons (B and C, see IZ).

(D) At higher magnification, radially oriented RGRS1 immunoreactive strands (arrowheads) that are aligned in parallel to cordons of neuronal nuclei (blue) are seen in cortical plate.

(E–G) Segments of radial glial processes in the cortical plate region, akin to those shown in (C) and (D), were labeled with anti-GFAP antiserum (E) RGRS1 mAbs (F) and superimposed to show localization of RGRS1 on radial glial processes (G).

(H) High-magnification view of RGRS1-positive strands (arrows) in the ventricular zone.

(I) Double immunohistochemistry for RGRS1 and GFAP show colocalization of RGRS1 to radial glial strands (red/orange, arrows) in VZ.

(J) Immunoelectron micrograph illustrates a segment of radial glial process in cortical plate immunoreactive for RGRS1 mAbs. Immunoreactive product (DAB) indicative of antibody binding is distributed on the surface of plasma membrane (arrowheads, I).

(K) In radial glia newly isolated from E16 cerebral wall, RGRS1 (red/orange) is primarily distributed at the top and bottom of the cells. Radial glial cell was colabeled with radial glia-specific anti-BLBP antibodies (green).

(L–O) In vitro, RGRS1 mAbs do not label postmigratory, differentiating cortical neurons (L and M) or migrating neurons (N and O). Cortical neurons were immunoreacted with polyclonal anti-MAP antibody (L) and RGRS1 mAbs (M). Migrating neurons (arrowheads; N) attached to glial strands (asterisk) do not label with RGRS1 mAbs (O).

(P) In adult cortex, RGRS1 mAbs label astroglial cells (arrows). These cells were colabeled with anti-GFAP antibodies (green).

Abbreviations: VZ, ventricular zone; IZ, intermediate zone; SP, sub plate; CP, cortical plate; MZ, marginal zone. Scale bars equal 50 μm in (A), 60 μm in (B), 70 μm in (C), 10 μm in (D), 12.5 μm in (E)–(G), 35 μm in (H) and (I), 1 μm in (J), 25 μm in (K), 35 μm in (L)–(O), and 45 μm in (P).

Signal molecule 1), recognizes an antigen that is predominantly distributed in a punctate manner at the superficial strata of the cortical plate, at the interface between the marginal zone and the newly developing cortical laminae, where neurons terminate their migration (Figures 1A–1C). In the developing rat cortex, RGRS1 immunoreactivity appears after the initiation of neuronal cell migration around embryonic day 13–14 (Figure 1A). By embryonic day 16, when great numbers of neurons are in the process of migration, RGRS1 immunoreactivity is distributed in a similar pattern at the top of the cortical plate (Figure 1B). Faint cell surface immunostaining of radially oriented processes is also noticeable in the ventricular zone. The intermediate zone, through which neurons migrate to reach the cortical plate, is immunonegative. By embryonic day 18, as the cortical plate (CP) expands, cell surface labeling of radially oriented processes in CP was observed (Figures 1C, 1D, and 1F). As at E16, RGRS1 is distributed in regions of the cerebral wall where neurons begin to

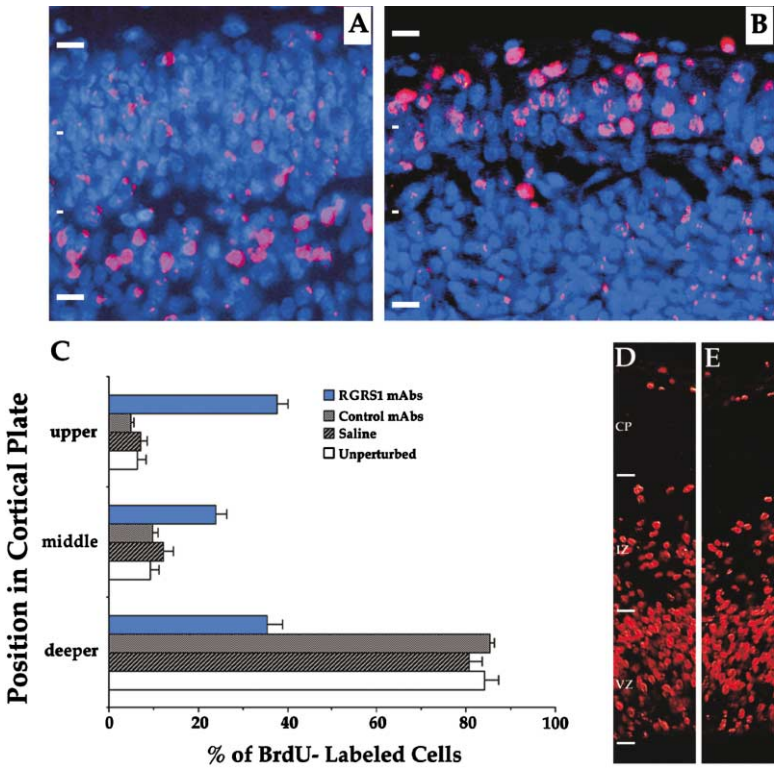

Figure 2. RGRS1 Antigen Regulates the Appropriate Termination of Neuronal Migration In Vivo

(A and B) Neurons generated on E15.5, destined to the deeper layers of cortical plate (CP), were labeled with BrdU. At E17, several hours prior to these labeled make their decision to terminate migration, Fab fragments of RGRS1 mAbs (1 mg/ml) were injected into lateral ventricles, and at E18, embryos were removed and the location of BrdU-positive cells in CP were analyzed. Panels (A) and (B) show cortical plate (CP) region (immunoreacted with anti-BrdU [red/pink] and counterstained with bis benzimide [blue]) of embryos injected with control mAbs (A) or RGRS1 mAbs (B). Under control conditions, BrdU-labeled cells ended their migration, detached, and settled into deeper layers of the cortical plate (A). Perturbation with RGRS1 mAbs caused most neurons to remain in ectopic locations, away from the deeper CP region (B). Bars in (A) and (B) indicate the top and bottom of cortical plate. Small bars indicate the division of CP into three equal regions (upper, middle, and deeper).

(C) Graph illustrates the location of BrdU-positive cells under different experimental conditions. Under control conditions (gray/white bars), most of the labeled neurons were found in deeper CP. However, when RGRS1 antigen was blocked, a greater proportion of labeled neurons were found in ectopic locations in upper and middle CP (blue bars).

(D and E) Control (D) or RGRS1 (E) antibody injection into the ventricles, while BrdU-labeled neurons (red) were migrating through the intermediate zone, did not affect normal neuronal migration.

Scale bars equal 20 μm in (A) and (B) and 35 μm in (D) and (E).

cease their migration and leave their radial glial scaffold. Immunoreactivity of radially oriented processes in the ventricular zone becomes more pronounced at this stage (Figures 1C, 1H, and 1I). Some of the transforming radial glial cells whose soma are in the ventricular zone might label with RGRS1 mAbs at this stage. The intermediate zone remains immunonegative at E18. Prominent labeling of the cortical plate and ventricular zone vestiges persists on neonatal day one. From neonatal day 7 onward, by which time neuronal migration to the cerebral cortex is already complete, immunoreactivity is primarily detected in the astrocytes (Figure 1P) and the ependymal layer lining the ventricles.

Double labeling studies with neuronal and glial markers indicate that the RGRS1 antigen is present on the radial glial cell surface, but not in neurons. In embryonic brain sections, segments of radially oriented processes at the top of the cortical plate (arrowheads, Figure 1D) or in ventricular zone (Figure 1I) labeled with both RGRS1 mAbs and radial glial-specific anti-GFAP antiserum (Figures 1E–1G and 1I). In immunoelectron microscopic analysis of embryonic cortical tissues, RGRS1 antibodies labeled the cell surfaces of longitudinally oriented, microtubule-rich, electronlucent, large diameter fibers (arrowheads, Figure 1J), all of which are characteristics of radial glial strands (Sidman and Rakic, 1973).

In vivo, and in vitro, RGRS1 mAbs immunolabel cortical radial glial cells, but not migrating neurons or postmigratory cortical neurons (Figures 1K–1O). In newly isolated radial glia, RGRS1 is primarily distributed at the top and bottom of the cells (Figure 1K), whereas in radial glia that were maintained without neuronal contact in vitro (>72 hr), RGRS1 is distributed uniformly (data not shown). Furthermore, when E17 cerebral cortical cells were immunolabeled live with RGRS1 mAbs, separated using a fluorescent activated cell sorter (FACS), cultured for 24 hr, and labeled with radial glial cell-specific rat-401 antibodies, 92% (±5.2%) of cells that were sorted on the basis of immunoreactivity for RGRS1 were positive for rat-401 immunoreactivity. Together, these results indicate that the RGRS1 antigen is expressed in radial glial cells both in vivo and in vitro and support the notion that its selective expression in glial strands spanning the developing cortical plate may enable it to influence the terminal phase of neuronal migration.

Function of RGRS1 Antigen In Vivo and In Vitro

To examine the role of RGRS1 as a putative signal in the termination of neuronal migration in vivo, we labeled a small population of cortical neurons with BrdU at their birth, allowed them to migrate to the cortical plate, and perturbed the function of the RGRS1 antigen with RGRS1 antibody fragments around the time these labeled neurons decide to end their migratory phase, and then we examined for effects on neuronal placement 24 hr later (Figure 2). In order to target neurons destined for cortical layers 5/6, pregnant rats at E15.5–E16 were given an intraperitoneal injection of BrdU and allowed

to develop another 36 hr. At E17, approximately 6 hr prior to BrdU-labeled neurons reaching the top of the cortical plate, purified Fab fragments of RGRS1 antibodies were injected into the lateral ventricle of the embryos using exo utero surgery. This allows for the antibodies to diffuse in and bind to the RGRS1 antigen in the cortical plate just as the BrdU-labeled neurons make their decision to terminate their migration. As a control, embryos at the opposite end of the uterine horn were sham injected with either saline or control antibody of the same isotype. Embryos in the middle of the uterus were left unperturbed.

After 24 hr, embryos were removed and the location of BrdU-positive cells in the dorsomedial regions of the occipital cerebral wall was analyzed. Brain sections were immunoreacted for BrdU and nuclei were counterstained with bis benzimide. Under control conditions, BrdU-labeled neurons appear to detach from their glial guides normally and settle into the deeper layers of the cortex (Figure 2A). Later generated, unlabeled neurons destined to the superficial layers migrate past them on their way to the top of the cortical plate. In embryos injected with the RGRS1 antibodies, BrdU-labeled neurons did not terminate their migration appropriately, and thus were distributed ectopically in the upper regions of the cortical plate (Figures 2B and 2C). To measure the effect of perturbation of RGRS1 antigen, the distribution pattern of BrdU-labeled cells in the developing cortical plate was analyzed. The cortical plate was divided into three equal sized bins: superficial, middle, and deeper, and the number of BrdU-positive cells in each of these regions was counted and plotted according to their location and different experimental conditions (Figure 2C). Under control conditions, 85% of the labeled neurons detached normally, appropriately settling into the deeper cortical layers. However, when the function of RGRS1 antigen was perturbed, most (65%) of the BrdU-labeled neurons remained ectopically in the upper or middle regions of the cortical plate. In control experiments, where RGRS1 antibodies were injected into the ventricles while the BrdU-labeled neurons were still in the process of migrating through the intermediate zone (at E16), no effect on normal neuronal migration was observed (Figures 2D and 2E). Immunostaining with glial-specific rat 401 monoclonal antibodies indicate that RGRS1 Fab fragment treatment does not affect the morphology or development of radial glial cells themselves (data not shown). Together, these data demonstrate that perturbation of the RGRS1 antigen in vivo specifically affects the terminal phase of neuronal migration and suggests that the RGRS1 antigen is a crucial signal in the appropriate termination of neuronal migration in the developing cerebral cortex in vivo.

How this antigen may function to derail migrating neurons from their radial glial guides once they have reached their proper location in the developing cortical plate remains unresolved in these in vivo studies. One possibility is that the RGRS1 antigen changes the adhesivity of radial glial cell surface at the top of the cortical plate, affecting the ability of neurons to continue their migration. To test this hypothesis, we performed short- and long-term neuron-glia aggregation assays, where dissociated neurons and glia from embryonic cerebral cortices (E16.5) were allowed to adhere to each other under

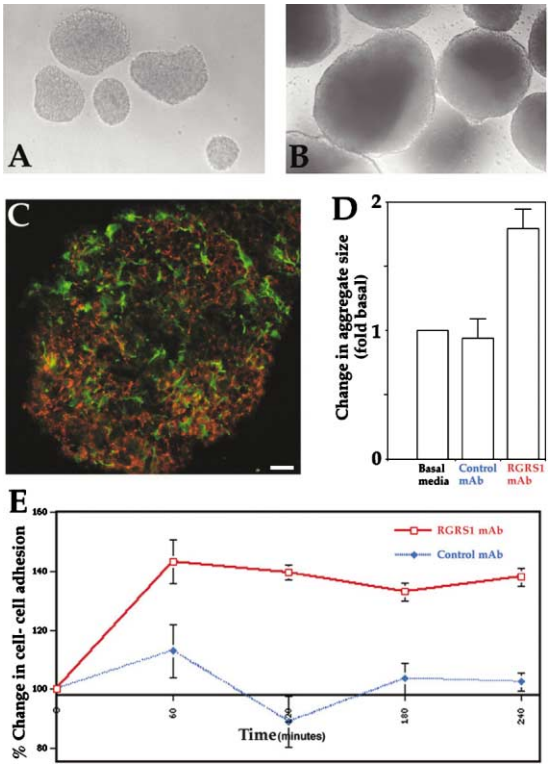

Figure 3. RGRS1 Is an Antiadhesive Molecule

(A and B) In long-term (24 hr) adhesion assays, cortical neurons and glia adhere to each other more readily and strongly and form larger aggregates in the presence of RGRS1 Fab fragments (B) than under control conditions (A).

(C) Neurons and glia adhered to each other throughout the aggregates. Aggregates were fixed, sectioned, and immunolabeled with neuron-specific anti-neuron-specific tubulin (Tuj-1) antibodies (red/orange), and astroglial-specific anti-GFAP antibodies (green).

(D) The average area of neuron-glia aggregates is increased by 74% in the presence of RGRS1 Fabs when compared with that of control conditions. The difference between RGRS1 and control groups are significant at $p < 0.05$ (Student's t test).

(E) In short-term aggregation assays, dissociated neurons and glia from embryonic cortices were allowed to adhere to each other in the presence of Fab fragments (100 μg/ml) of control or RGRS1 antibodies. After 60, 120, 180, and 240 min of incubation, changes in cell-cell attachment were measured. Inhibition of RGRS1 significantly increased cell-cell attachment. Exposure to a control antibody of the same isotype did not significantly alter cell-cell attachment. In both long-term and short-term adhesion assays, inhibition of RGRS1 lead to enhanced cell-cell attachment, suggesting that RGRS1 is an antiadhesive molecule.

Scale bars equal 90 μm in (A) and (B) and 10 μm in (C).

control conditions or in the presence of function-perturbing RGRS1 Fab fragments (100 μg/ml) for 24 hr or shorter (Figures 3A and 3B). In long-term adhesion assays (24 hr), under control conditions, neurons and glia adhere to each other and form small aggregates (Figure 3A). However, in the presence of RGRS1 antibodies, neurons and glia form significantly larger aggregates, suggesting that RGRS1 antigen is an antiadhesive molecule, in the absence of which neurons and glia adhere to each other more readily and strongly (Figure 3B). A control antibody of the same isotype (IgM) to a ubiquitous cell surface molecule had no effect on the

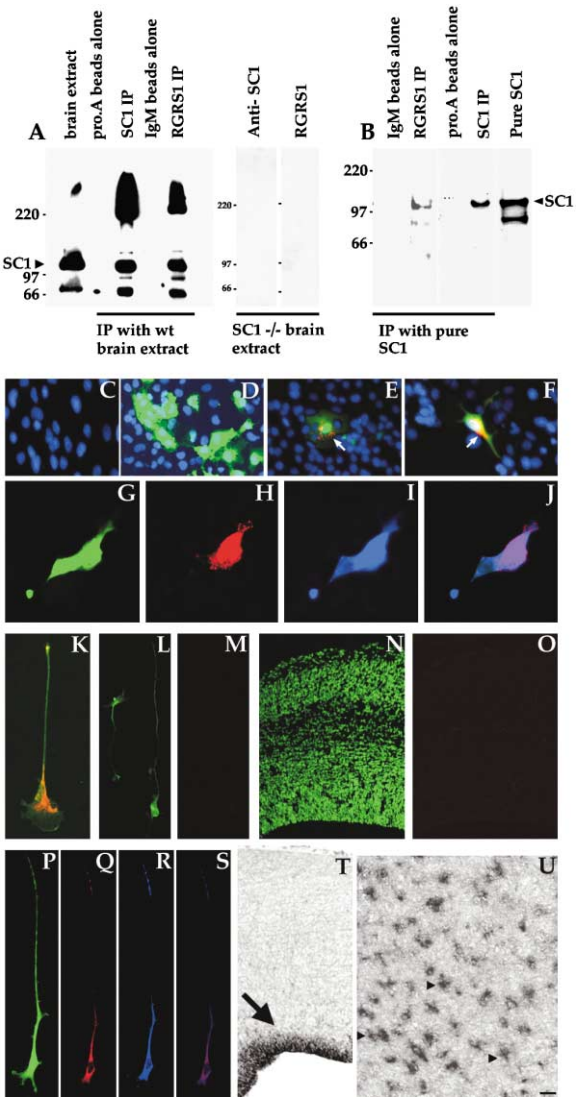

Figure 4. RGRS1 Antigen Is SPARC-like 1

(A) Immunoprecipitation of SC1 from mouse brain extract (A) and purified, recombinant SC1 protein (B). SC1 was immunoprecipitated from mouse brain extracts with both anti-SC1 and RGRS1 antibodies (A). The lower molecular weight bands below the SC1 band are likely due to degradation during the immunoprecipitation, whereas the higher molecular weight (~220 kDa) species are likely to be modified SC1. None of these protein bands were found in SC1 null brains.

(B) Purified, recombinant SC1 is IPed by anti-SC1, as well as RGRS1 antibodies.

(C–J) Cos-7 cells were transfected with pIRES-EGFP or pIRES-SC1-EGFP, immunostained with RGRS1 or anti-SC1 antibodies, and nuclear counterstained with bis benzimide (blue).

(C) Untransfected Cos-7 cells probed with the RGRS1 antibody reveals no labeling.

(D) Cos-7 cells transfected with pIRES2-EGFP and probed with the RGRS1 antibody also reveals no labeling.

(E and F) pSC1-EGFP transfected Cos-7 cells probed with the RGRS1 antibody reveals RGRS1 immunoreactivity (red/yellow, arrow) of the EGFP-expressing cells (green).

(G–J) Anti-SC1 and RGRS1 immunoreactivity were localized to the same cell. Cos-7 cells were transfected with pSC1-IRES-EGFP, and EGFP expression (G), RGRS1 immunoreactivity (H), and anti-SC1 immunoreactivity (I) were colocalized to the same cell (J).

(K) RGRS1 immunoreactivity in radial glia (red/orange) colabeled with radial glia-specific anti-BLBP antibodies (green).

size of the aggregates formed (Figure 3D). In short-term cell-cell adhesion assays, where dissociated embryonic cortical cells were allowed to adhere to each other in the presence of Fab fragments of control or RGRS1 antibodies for 60, 120, 180, or 240 min, inhibition of the RGRS1 antigen significantly increased cell-cell attachment, further confirming that RGRS1 is an antiadhesive molecule (Figure 3E). Exposure to Fab fragments of a control antibody of the same isotype did not significantly alter cell-cell attachment. The ability to inhibit cortical cell adhesion in vitro, together with the perturbation studies in vivo, suggests that the RGRS1 antigen could function to derail neuronal migration by reducing the adhesivity of neurons to the radial glial cell surface at the top of the CP during the terminal phase of neuronal migration.

Characterization of RGRS1 Antigen as SPARC-like 1

Once the functional significance of the RGRS1 antigen in the appropriate termination of glial-guided neuronal migration in the cerebral cortex was established, we sought to identify the molecular nature of the RGRS1 antigen. We screened an E16 mouse brain expression library, using the RGRS1 antibody as a probe. 1×10^6 transformants were screened, and 30 potential positive clones were identified initially. Upon subsequent tertiary screening, two identical clones were isolated, which upon sequence analysis were identified as mouse SPARC-like 1 (SC1). Both clones contained full-length cDNA inserts with an ~2 kb ORF. Mouse SC1 is a 650 amino acid protein with an apparent molecular weight of 116/120 kDa (Johnston et al., 1990), which shares homology with members of the SPARC family of extracellular matrix proteins.

In order to confirm that the RGRS1 antibody recognizes SC1, the RGRS1 antigen in E16 mouse brain extract was immunoprecipitated with RGRS1 antibody or an SC1-specific monoclonal antibody, and then immunoblotted with SC1-specific antibody. Anti-SC1 antibodies recognized identical bands from the RGRS1 and SC1 immunoprecipitation reactions, which corresponds to the SC1 protein (Figure 4A). In addition, both RGRS1 and anti-SC1 antibodies immunoprecipitate purified recombinant SC1 protein (Figure 4B). Furthermore, immu-

(L and M) Presorption of the RGRS1 antibody with SC1 protein abolishes RGRS1 labeling (M). Cells shown in (M) were colabeled with anti-BLBP antibodies (green, L).

(N and O) RGRS1 antibodies do not label E17.5 SC1 mutant cortex (O). Cortical sections were nuclear counterstained with Neurotrace 500 (N).

(P–S) In GFP expressing E16 embryonic radial glia from hGFAP-GFP mouse cortex (P), RGRS1 (Q) and anti-SC1 (R) immunoreactivity was colocalized (S; merged image of Q and R).

(T and U) In situ hybridization using probes directed against SC1 reveals SC1 transcripts are abundant in the ventricular zone of the E16.5 cerebral cortex, where the radial glial cell bodies are located (T). Arrows indicates the ventricular zone region. (U) Multipolar astroglial cells (arrowheads) label prominently in mature mouse cortex. Scale bars equal 25 μm in (C)–(F) and (K)–(M), 5 μm in (G)–(J), 70 μm in (N) and (O), 30 μm in (P)–(S), 60 μm in (T), and 45 μm in (U). Positions of molecular weight markers (kDa) are indicated on the left in (A) and (B).

noblots of brain extracts from SC1 null mice displayed no RGRS1 or anti-SC1 immunoreactivity (Figure 4A). Together, these data indicate that the SC1 protein is the antigen recognized by the RGRS1 antibody.

To further confirm the identity of RGRS1 antigen as SC1, we transiently transfected Cos-7 cells with an SC1-EGFP-expressing plasmid (pSC1-IRES-EGFP) and then assayed transfected cells for SC1 or RGRS1 immunoreactivity. Double labeling experiments revealed that EGFP-positive cells, indicating the expression of SC1-EGFP, were also found to be immunoreactive with either the SC1 or the RGRS1 antibodies (Figures 4E and 4F). Triple labeling experiments demonstrated that cells expressing SC1-EGFP were positive for both SC1 as well as RGRS1 immunoreactivity (Figures 4G–4J). Control experiments in which anti-SC1 primary antibody-labeled cells were incubated with IgM-specific secondary antibodies and RGRS1 primary antibody-labeled cells were incubated with IgG-specific secondary antibodies did not generate any labeling, indicating that the results obtained in the triple labeling experiments were not due to cross-reactivity of the secondary antibodies to their cognate primary antibodies (not shown). Furthermore, RGRS1 antibody labeling of SC1-EGFP-expressing cells was abolished when RGRS1 antibodies were presorbed with pure SC1. Cells expressing control EGFP or untransfected cells did not label with anti-SC1 or RGRS1 antibodies (Figures 4C and 4D). Radial glial immunoreactivity of RGRS1 mAbs was abolished when RGRS1 antibodies were presorbed with recombinant SC1 protein (Figures 4K–4M). RGRS1 immunoreactivity is absent in SC1-deficient embryonic cortex (Figures 4N and 4O). In addition, newly isolated primary radial glial cells from E16 mouse cortex colabel with anti-SC1 and RGRS1 antibodies (Figures 4P–4S). Together these data indicate that SC1 expression can be detected by both SC1 and RGRS1 antibodies and supports the notion that RGRS1 recognizes SC1 in vivo.

Since RGRS1 antibodies immunolabel radial glial cells and astrocytes in the developing and mature cerebral cortex, we determined the regional and cellular localization of SC1 expression using in situ hybridization analysis. In embryonic CNS, SC1 transcripts are most abundant in the ventricular zone of the spinal cord and cerebral cortex, where the radial glial cell bodies are located, and in mature cortex, SC1 is expressed in astrocytes (Figures 4T and 4U).

Together, data from cDNA library screen, immunoprecipitation analysis, SC1-EGFP transfection assays, SC1 null mice, and in vivo cellular localization studies suggest that RGRS1 antibodies recognize SPARC-like 1 (SC1).

SC1 Inhibits Adhesion of Cortical Neurons In Vitro

We then sought to determine the effect of SC1 on the adhesion of embryonic cortical neurons in vitro. A radial glial cell line, C6R (Friedlander et al., 1998), or Cos-7 cells were transfected with pSC1-IRES-EGFP or a control pIRES-EGFP plasmid and cultured for 24 hr. Dissociated E16 cortical neural cells were then added to transfected cultures. The cocultures were incubated an additional 24 hr, then fixed and labeled with neuron-specific Tuj-1 antibodies and assayed for the number of EGFP-positive cells in contact with Tuj-1-positive neurons. In C6R cells expressing pSC1-IRES-EGFP, only 12% of SC1-EGFP-positive cells were in contact with a Tuj-1-positive cortical neuron, whereas in control cultures transfected with the pIRES-EGFP plasmid, 45% of EGFP-positive cells were in contact with a Tuj-1-positive cortical neuron (Figures 5C, 5D, and 5H). Often observed in the pSC1-IRES-EGFP transfected cultures were Tuj-1-labeled cells in proximity to an EGFP-positive cell, but processes from the cortical neurons avoided the SC1-EGFP-expressing cell, skirting its perimeter. Similar results were obtained with COS-7 cells expressing pSC1-IRES-EGFP (Figures 5A and 5B). In these cultures, 8% of SC1-EGFP-positive cells were in contact with a Tuj-1-positive cortical neuron, while in control cultures transfected with pIRES-EGFP, 57% of EGFP-positive cells made contacts with cortical neurons (Figure 5H).

Adhesivity of embryonic cortical neurons was also tested on primary radial glial cells expressing SC1. Dissociated E16 cortical cells were transfected with pSC1-IRES-EGFP or pIRES-EGFP, and after 72 hr in vitro, fixed and labeled with anti-GFAP and Tuj-1 antibodies. The number of EGFP- and GFAP-positive radial glial cells in contact with Tuj-1-positive neurons in the cultures was measured. Similar to the results obtained with Cos cells and C6R cells, primary cortical glial cells transfected with control EGFP were found to be in contact with neurons more frequently than glial cells expressing SC1-EGFP (EGFP: SC1-EGFP, 58%: 14%; Figures 5E–5H).

SC1 Inhibits Adhesion of Dissociated Cortical Cells to Cortical Slices

To further characterize SC1's antiadhesive function, we next assessed the effect of exogenous SC1 protein on the adhesion of embryonic cortical cells. Dissociated, CMTMR-labeled, E14 cortical cells were seeded on E16 cortical slices, and the combined cultures were maintained in either basal media or media containing purified SC1 protein, SC1 protein + RGRS1 antibody, RGRS1 antibody alone, or a control antibody for 24 hr. Cultures were then analyzed for adhesion of CMTMR-labeled cells to cortical slices. On slices that were maintained in untreated media, dissociated cortical cells adhered well throughout the cerebral wall (Figure 5I). However, on slices maintained in media containing SC1 protein, only a sparse population of cortical cells were adherent (Figure 5J). Preincubation of the SC1 protein with RGRS1 antibodies abrogated the antiadhesive effects of the SC1 protein, such that after 24 hr in culture, a large number of cortical cells were found adherent to the cortical slices (Figure 5K). Control antibodies alone had no effect as compared to the untreated control, while addition of the RGRS1 antibodies caused a moderate increase in adhesivity of cortical cells (Figure 5L). This is probably due to the binding of the RGRS1 antibody to endogenous SC1 both on the slices as well as the dissociated cells, thereby blocking its antiadhesive effects, resulting in an increase in total number of bound cortical cells. Together, these data demonstrate that SC1 significantly decreases the adhesivity of developing cortical cells and that RGRS1 antibody neutralizes this effect.

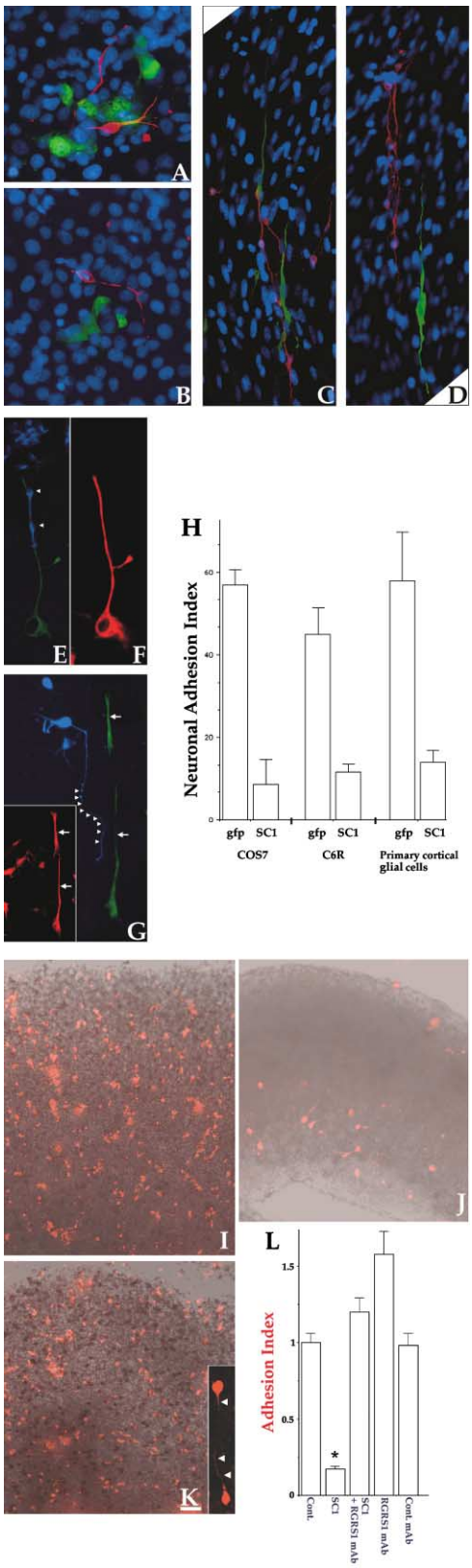

Figure 5. SPARC-like 1 Inhibits Neuronal Adhesion In Vitro

(A–D) Cortical neurons (red) avoided adhering to or contact with SC1-GFP-expressing Cos cells (B) or C6R cells (D), but not with control GFP-expressing cells (A and C). Nuclei of the cells were counterstained with bis benzimide (blue).

SC1 Misexpression in the Developing Cerebral Wall Disrupts Neuronal Migration In Vivo

Since our perturbation experiments using anti-SC1 antibodies and recombinant SC1 disrupted neuronal placement in vivo and neuronal cell adhesion in vitro, we sought to determine the effect of ectopic expression of SC1 on cell migration in the cortex in vivo. We ventricle injected and then electroporated E15.5 mouse cortices with either pSC1-IRES-EGFP or the control plasmid pIRES-EGFP. Cortical slices from electroporated brains were cultured for 24 hr. BrdU was then added to the media and slices were maintained for an additional 24 hr. A large number of migrating neurons are transfected in this assay and we monitored the extent of migration of SC1-GFP- or GFP-positive neurons into the cerebral wall. Overexpression of SC1 clearly disrupted neuronal cell migration into the cerebral wall (Figure 6). Quantification of the distance of leading cells from pial surface (normalized to the full length of cerebral wall) indicates GFP control neurons migrated 77.8% ± 2.7% of the length of the cerebral wall. In contrast, SC1-expressing neurons migrated only up to 30.22% ± 2.6% of the length (Figure 6E). Migration of untransfected, BrdU-positive cells into the cerebral wall was also retarded following SC1 expression (Figure 6F). This may have resulted from the association of these untransfected neurons with SC1 overexpressing radial glia. When SC1-GFP- and GFP-expressing neurons were monitored in real time, GFP neurons migrated actively in the cerebral wall at a rate of 27 ± 2.4 μm/hr, displaying the characteristic saltatory somal movement, as well as active leading and trailing processes. In contrast, SC1 expression significantly retarded the rate of migration (7.6 ± 1.1 μm/hr), and these neurons do not display characteristic motile behavior (Figure 7, also see Supplemental Movie at http://www.neuron.org/cgi/content/full/41/1/57/DC1).

(E–G) Primary embryonic cortical cultures were also transfected with pIRES-EGFP (E) or pSC1-IRES-EGFP (G). Neurons (arrowheads) attached to GFP-expressing glial cells (E), whereas they avoided SC1-GFP-expressing glia (arrows, G). Radial glial cells expressing EGFP (green) were identified by GFAP (red) colocalization (F, inset in G), and neurons (blue) were identified by Tuj-1 immunoreactivity. Arrowheads in (G) indicate neuronal process.

(H) Number of neurons in contact with or adhering to EGFP-positive cells was quantified from four independent experiments. In each example, significantly fewer neurons contacted or adhered to SC1-EGFP-expressing cells. Number of EGFP-positive cells studied in each category is >400.

(I–K) Purified SC1 blocks adhesion of dissociated neuronal cells to cortical slices. CMTMR-labeled, E14 cortical neuronal cells (red) were seeded on E16 cortical slices and cultured in basal media (I), SC1-supplemented media (J), or in RGRS1 antibodies presorbed with 10 μg/ml SC1 protein (K). Under control conditions, CMTMR-labeled cells attach to slices and extend processes (arrowhead, inset K). SC1 significantly reduces the adhesion of dissociated cortical cells to cortical slices. This effect was abrogated when SC1 was presorbed with RGRS1 antibodies. RGRS1 antibodies alone caused an increase in adhesion, whereas control antibodies had no effect.

(L) The histogram represents the average of three experiments in which at least 30 cortical slices in each condition were analyzed for the number of adherent CMTMR-labeled cells, using the Metamorph software program.

Scale bars equal 20 μm in (A), (B), and (E)–(G); 60 μm in (C) and (D); and 90 μm in (I)–(K).

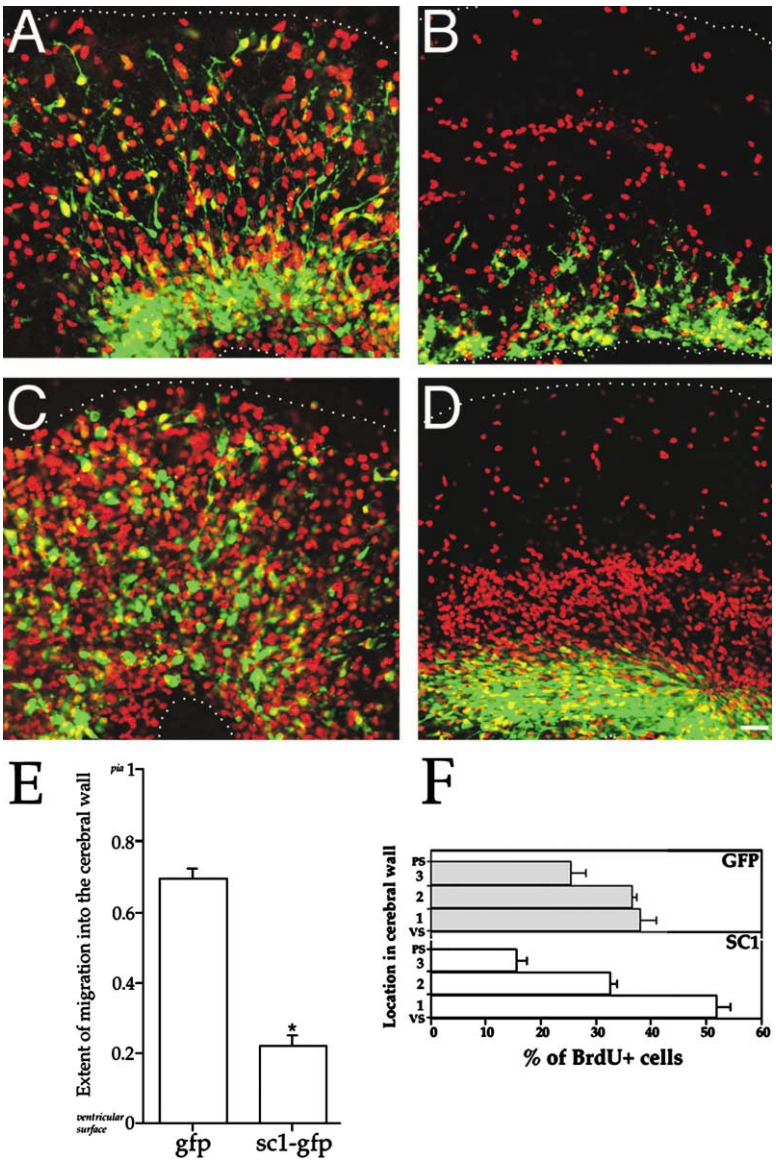

Figure 6. SPARC-like 1 Inhibits Neuronal Migration

(A–D) Ventricular zone of embryonic day 15.5 mouse cerebral cortices were electroporated with cDNAs encoding GFP (A and C) or SC1-GFP (B and D). Cortices were then sectioned and slices were maintained for 24 hr. BrdU was then added to the media, and after 24 hr, slices were labeled with anti-BrdU antibodies (red). GFP-labeled neurons migrated into the cortical plate (A and C), whereas the migration of cells expressing SC1-GFP was greatly reduced (B and D).

(E) Quantitative analysis of cell migration into the cerebral wall indicates that GFP-expressing cell migrated 77.8% of the length of the cortex, whereas SC1-GFP-positive cells migrated only up to 30% of the length of the cortex.

(F) Fewer BrdU-positive cells (red) migrated into the upper regions of cortex in SC1-expressing slices. To quantify BrdU cell distribution, cerebral wall was divided into three equal bins from ventricular surface (VS) to pial surface (PS), and the percentage of BrdU-positive cells in each area was counted. Number of slices quantified: GFP, 32; SC1-GFP, 31. Data shown are mean ± SEM; asterisk, significant when compared with controls at $p < 0.01$ (Student's t test). Dotted lines indicate the pial (top) and ventricular (bottom) surfaces of the slices.

Scale bar equals 22.5 μm in (A) and (C) and 30 μm in (B) and (D).

These studies, together with previously described adhesion assays, demonstrate that antiadhesive activity of SC1 inhibits neuronal migration in vivo and suggests that SC1 may help terminate neuronal migration appropriately.

Cortical Laminar Organization in SC1 Mutant Mice

The characteristic laminar architecture of the cerebral cortex in SC1 nulls appears not to have been altered significantly in Nissl-stained sections (Figure 8).

To carefully investigate the pattern of cortical neuronal placement in SC1-deficient mice, we labeled newly generated neurons with BrdU and analyzed their placement in the mature cerebral cortex. When BrdU was injected at E16, BrdU-labeled cells were confined primarily to the upper layers of the wild-type cortex, whereas in mutant cerebral cortex, labeled cells were distributed consistently within a wider region in the upper cortex (Figure 8). Measurement of the width of BrdU-labeled cell distribution (normalized to the entire width of cortex) indicates that there is a 1.7-fold increase in the width of BrdU cell distribution in mutants (wt = 0.127 ± 0.013, −/− = 0.221 ± 0.03). Similarly, analysis of BrdU+ cell position in cerebral cortex indicates the wider distribution of BrdU+ cells in SC1 null mice (Figure 8E). This trend of ectopic neuronal placement in SC1 null mice further supports a role for SC1 in the appropriate placement of neurons in cerebral cortex.

Discussion

We have identified a radial glial antigen (RGRS1) whose expression and function suggest that it may provide a "release" signal that initiates the conversion from the migratory to postmigratory state in the developing cortex. Molecular and biochemical analyses demonstrate that the RGRS1 antigen is SPARC-like 1 (SC1), a member of the SPARC family of counteradhesive extracellular

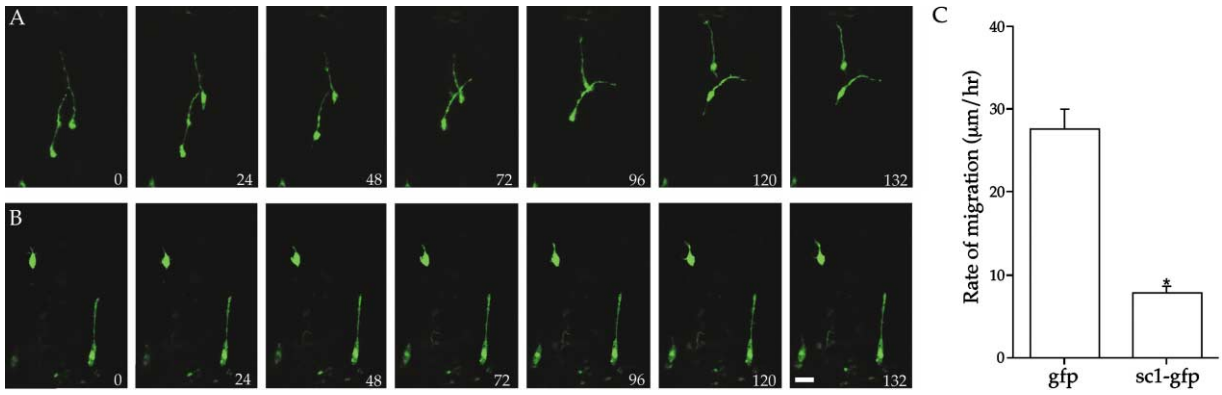

Figure 7. Real-Time Analysis of the Effect of SC1 on Neuronal Migration

GFP- (A) or SC1-GFP- (B) expressing cells in the intermediate zone of the transfected cortical slices were repeatedly monitored every 6 min. GFP-expressing cells migrated robustly at an average rate of 27 ± 2.4 μm/hr, whereas SC1-GFP-expressing cells migrated only at a rate of 7.6 ± 1.1 μm/hr (C). Number of cells analyzed: GFP, 106; SC1-GFP, 89. Data shown are mean ± SEM; asterisk, significant when compared with controls at $p < 0.01$ (Student's t test). Time elapsed since the beginning of observations are indicated in minutes. Scale bar equals 30 μm. Also, see the Supplemental Movie at http://www.neuron.org/cgi/content/full/41/1/57/DC1.

matrix proteins. Expression of SC1 on radial glial migratory pathways at the top of the CP, as well as it antiadhesive activity in vivo and in vitro, suggest that it may function to terminate glial-guided neuronal migration by reducing the adhesivity of neurons to the radial glial cell surface, thereby facilitating their detachment and aggregation into specific layers of the cerebral cortex.

Function of SPARC Family of Proteins

SC1 is an extracellular matrix-associated protein that shares 53% amino acid identity and functional similarity with SPARC (secreted protein acidic and rich in cysteine), an antiadhesive matricellular glycoprotein (Johnston et al., 1990; Soderling et al., 1997). The SPARC family of proteins, which includes testican, tsc36/FRP, QR1, SRG, SPARC-like 1/SC1/hevin, and SPARC itself, is defined by the presence of a follistatin-like domain and an extracellular calcium (EC) binding domain (reviewed in Brekken and Sage, 2000; Yan and Sage, 1999). The follistatin-like domain, which shares homology to a repeated domain in follistatin, consists of a Kazal-type serine protease inhibitor region and an EGF domain. The EC binding domain contains a canonical pair of Ca^{2+} binding EF-hand motifs (Yan and Sage, 1999). The N-terminal domains of each family member exhibit the greatest divergence, and testican and tsc36/FRP both contain additional domains at their C termini not present in the other members of SPARC family (Yan and Sage, 1999).

Both structural and functional analyses of SC1 suggest that it could influence the terminal phase of neuronal migration in multiple ways. For example, SC1/hevin has been shown to inhibit attachment and spreading of endothelial cells on fibronectin in vitro (Girard and Springer, 1996). Our data demonstrate that embryonic cortical neurons do not adhere to and avoid contact with SC1-expressing Cos cells, C6R radial glial cells, and cortical radial glial cells. Recombinant SC1 significantly reduced adhesion of cortical neural cells to live embryonic cortical slices. Furthermore, ectopic expression of SC1 inhibits neuronal migration. BrdU birthdating studies indicate a moderate but consistent neuronal displacement in SC1-deficient cerebral cortex. One might have expected cortical neurons to overshoot their target layer and migrate ectopically, into the marginal zone, following the loss of antiadhesive SC1. However, SC1 null mice do not exhibit such large-scale, gross developmental defects in the brain (McKinnon et al., 2000), perhaps due to the coincident expression of functionally similar molecular cues (McKinnon et al., 1996; McKinnon and Margolskee, 1996; Mendis et al., 1996; Johnston et al., 1990). Whether the neuronal displacement in $SC1^{-/-}$ is due to premature arrest of migration or enhanced adhesion or is indirectly influenced by disregulation of other neuronal placement and differentiation cues in the cortical plate remains to be determined. Nevertheless, these data support a model whereby SC1 facilitates a localized decrease in neuronal adhesivity at the top of the cortex by enabling the disassembly of cell adhesion contacts, and thus leading to the initiation of neuronal aggregation into distinct cortical layers.

Alternatively, SC1, like its close relative SPARC, could remodel the ECM in the developing cortical plate by modulating the production and activity of matrix metalloproteinases (Gilles et al., 1998) or by binding and sequestering Ca^{2+} through its EC domain or through its association with ECM components such as vitronectin and collagen types I, III, IV, and V (Maurer et al., 1995; Sasaki et al., 1998). Our biochemical analysis indicates that in addition to native SC1 protein at ~120 kDa, a high molecular weight protein of ~220 kDa can be immunoprecipitated from embryonic mouse brain extract by both the RGRS1 and anti- SC1 antibodies. Both antibodies also immunoprecipitate smaller degradation products of the intact SC1 protein (Johnston et al., 1990; Hambrock et al., 2003). None of these protein complexes were found in SC1 null brains. The identification of the ~220 kDa protein recognized by SC1 antibodies sug-

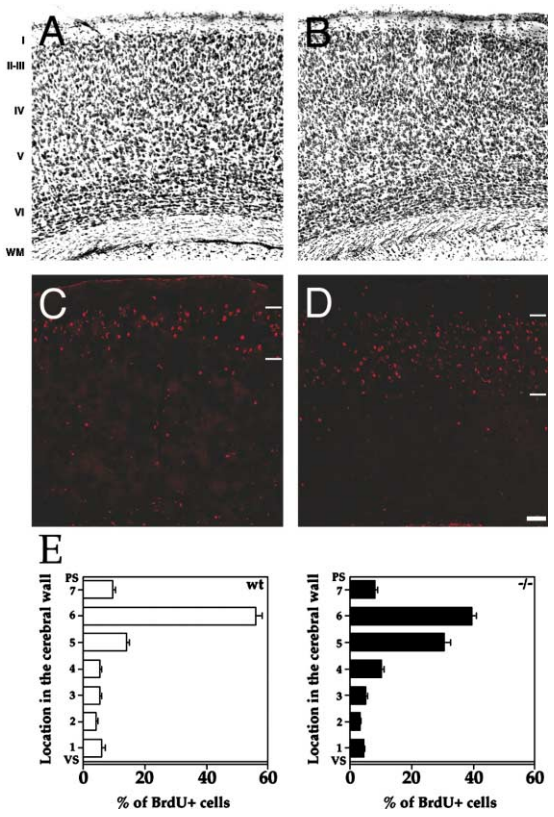

Figure 8. Cortical Laminar Organization of SC1-Deficient Mice

(A and B) Sections of the cerebral cortex from 6-week-old wild-type (A) and SC1-deficient (B) mice were Nissl stained. Differences in the organization of SC1-deficient mice cortex are not readily apparent. (C and D) Neurons that are destined to the upper layers of cerebral cortex were labeled at their birth (E16) with BrdU and their location was analyzed at 6 weeks after birth. BrdU-labeled neurons were found in the upper layers of cerebral cortex in wild-type mice as expected (C). However, in SC1-deficient cortex, BrdU-labeled neurons were present diffusely in the upper regions of cortex (D).

(E) To quantify BrdU cell distribution, cerebral cortex was divided into seven equal bins from ventricular surface (VS) to pial surface (PS), and the percentage of BrdU-positive cells in each area was measured. This indicates the ectopic placement of neurons in SC1-deficient cortex.

Small bars in (C) and (D) indicate the approximate width of BrdU+ cell distribution. Data shown are mean ± SEM. BrdU+ cells were counted in ten sections of the cerebral cortex from 5 embryos each of wt and SC1 mutant mice. Scale bar equals 60 μm.

gest that SC1 may exist in vivo as a highly glycosylated proteoglycan, or in strong association with ECM molecules such as collagen I (Hambrock et al., 2003) or other yet to be identified proteins. The nature and the function of SC1's association with other proteins, its posttranslational modifications, and its proteolytic processing during corticogenesis remains to be elucidated. However, the significance of the extracellular matrix protein assembly and remodeling for the developing cortex is evident in the disrupted corticogenesis seen in mice deficient in the ECM components perlecan (Costell et al., 1999), laminin α_5 chain (Miner et al., 1998), or laminin γ_1 nidogen binding site (Halfter et al., 2002) and in humans with secondary deficiencies in basal lamina assembly (Olson and Walsh, 2002).

Radial Glial Cell Function during Cortical Development

Two distinct radial glial developmental events guarantee the emergence of laminar organization of neurons in cerebral cortex: the generation of a radial glial cell scaffold that provides primary support for neurogenesis and migration and the coordinated sequence of glial-guided neuronal migration (reviewed in Kriegstein and Gotz, 2003; Rakic, 2003). Comparative analysis of radial glial cell surface cues involved in neuronal migration or neuron-radial glial interactions indicates that radial glial strands spanning the cerebral wall do not present a uniform pathway, providing merely passive support for migration. Rather, temporal and spatial changes in the distribution of cell surface cues along radial glial guides can actively influence neuronal differentiation and placement during corticogenesis. Our studies show that the antiadhesive radial glial cell surface-based molecule, SPARC like-1 (SC1), is primarily localized to the region of the radial glial processes spanning the cortical plate where neuronal migration on radial glial guides must end, and to a lesser extent, in the basal, cell soma region spanning the ventricular zone where premature onset of migration has to be avoided. Thus, SC1 is appropriately expressed, both temporally and spatially, to act as a termination signal for neuronal migration in the developing cerebral cortex. Whether the developmental expression of SC1 in the astroglial cells of cerebellum and hippocampus serves a similar function in these regions remains to be elucidated (Mendis et al., 1996; Mothe and Brown, 2001).

The function of SC1 as a terminator for neuronal migration depends on its specific deployment to appropriate locations along the radial glial migratory pathway in the developing cerebral cortex. Lack of SC1 expression in the intermediate zone of the cerebral wall, where most neuronal migration occurs, is reminiscent of expression patterns of other migration inhibiting molecules such as slit, expressed in regions surrounding the migratory pathways of developing telencephalon (Wu et al., 1999; Hu, 1999). In vitro, asymmetric cellular localization of RGRS1 in radial glia appears to depend on neuron-derived signals. Cortical neuronal cues are known to play a determinant role in radial glial maintenance and function. For example, Neuregulin-1, expressed by migrating neurons, promotes cortical neuronal migration on radial glia and concurrently supports the lengthening of the radial glial processes (Anton et al., 1997). Similarly, reelin can also influence radial glial morphology (Hartfuss et al., 2003). Specialized cell attachments between migrating neurons and radial glia, mediated via neuronal astrotactin, is thought to be crucial in initiating and maintaining neuronal cell migration (Hatten and Mason, 1990; Zheng et al., 1996). Thus, signals emanating from neurons such as neuregulin-1, reelin, or astrotactin may function to influence distinct regionalization of SC1 into functionally appropriate locations on the radial glial cell surface in the developing cerebral wall.

The functional significance of SC1 expression in the VZ during neuronal migration remains to be characterized. However, it may provide a mechanism for the coordination of neural precursor proliferation in the VZ and neuronal placement in the cortical plate or for the prevention of premature onset of migration.

Although the laminar identity of a neuron seems to be determined at its birth (McConnell, 1990; Valcanis and Tan, 2003), the diversity of molecules that are known to influence the final positioning of neurons in cerebral cortex (i.e., reelin, VLDLR, ApoER2, dab1, integrins, cdk5, p35, doublecortin, NT-4, calcium; reviewed in Marin and Rubenstein, 2003; Olson and Walsh, 2002; Komuro and Rakic, 1996) suggests that how neurons navigate to their appropriate location is likely to involve a complex orchestration of changes in cell-cell recognition, cell adhesion, and cell motility events. Signals to end neuronal migration are thought to arise from cell type- and context-dependent combinatorial interactions of multiple sources; for example, from reelin released by marginal zone Cajal-Retzius cells, from cell-cell recognition with other neurons in the appropriate laminar destination, or from interactions with incoming afferent thalamic axons (D'Arcangelo et al., 1995; Frantz and McConnell, 1996; McConnell, 1990; McConnell and Kaznowski, 1991; Marin and Rubenstein, 2003; Hatten and Mason, 1990; O'Rourke et al., 1992; Rakic, 1972; Rakic and Caviness, 1995). Identification of a role for SC1 in this process reveals a hitherto undefined mechanism at work in determining how motile neurons make their decision to end at appropriate locations in the developing cerebral cortex. Changes in the adhesivity of the radial glial cell surface due to the presence of antiadhesive cues such as SC1 at the top of the CP defines a novel mechanism whereby radial glial cells influence the final positioning of neurons in the cerebral cortex.

Experimental Procedures

In Vivo Assay to Evaluate the Function of RGRS1 Antigen in Neuronal Migration

Pregnant rats (Charles River) were injected intraperitoneally with BrdU (7.5 μg/gm body weight) on embryonic day 15.5–16. At E17 (36 hr past BrdU injection), when the labeled neurons reach the cortical plate, purified Fab fragments of antibodies (1 mg/ml) were injected into the lateral ventricles using exo utero surgical techniques (Muneoka et al., 1990). Embryos in one end of the uterine horn received RGRS1 antibody injection, whereas the embryos in the other end of the uterine horn received either saline or control antibody injection. Embryos in the middle were left unperturbed.

At E18, embryos were removed and the location of BrdU-positive cells in the dorsomedial regions of the occipital cerebral wall was analyzed as follows. 10 μm thick, coronal paraffin sections from rostral to caudal end of occipital cortex of occipital cortex were processed for BrdU immunohistochemistry and bis benzimide counter staining (Anton et al., 1999). The location of BrdU-positive neurons in alternate sections was quantified by dividing the cortical plate into three equal strata (superficial, middle, and deeper) using the NIH Image 1 program and counting the number of most heavily labeled neurons in each of these divisions.

As a control for specificity of the effect, some animals received BrdU injection (50 μg/gm body weight) and antibody infusion on the same day. 24 hr later, brains were removed and processed for BrdU labeling to evaluate the migration pattern of neurons through the intermediate zone. Fab fragments were generated using Immunopure IgM fragmentation kit (Pierce).

Aggregation Assay

Cerebral cortices from E16–E17 embryos were removed and dissociated as described earlier (Anton et al., 1999). Cells were plated at 300K cells/2 ml in 24-well dishes and rotated at 90 rpm in a 37°C (95% O_2/5% CO_2) incubator for 2 to 24 hr. Wells were supplemented with 100 μg/ml RGRS1 or control mAbs (Fab fragments) or left unsupplemented. At the end of incubation, the number of unattached, living single cells in the suspension was counted using trypan blue exclusion method. Change in cell-cell aggregation was calculated as follows: ([number of aggregated cells in media supplemented with antibodies/number of single cells seeded originally]/[number of aggregated cells in unsupplemented media/number of single cells seeded originally] × 100). In long-term assays (24 hr), all the aggregates were harvested and their diameter was measured using the NIH Image 1 program.

cDNA Library Screening

An embryonic day 16 mouse brain expression library was constructed in λZAP Express EcoR I/Xho I (Stratagene) according to manufacturer's instructions. Titer of the library is 2×10^9 pfu/ml. For screening, the library was plated at 50,000 pfu. IPTG saturated Hybond-C nitrocellulose filters with orientation marks were used to make plaque lifts. Filters were then processed for RGRS1 immunoreactivity with RGRS1 hybridoma supernatants. Plasmids from positive plaques, following tertiary screen, were recovered according to manufacturer's directions.

Immunoprecipitation

E16 mouse brains were homogenized in RIPA buffer (2.0 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% Na-Deoxycholate, 5% Glycerol) containing 1× complete protease inhibitor cocktail (Roche) and 1 mM PMSF, then cleared by centrifugation. The resulting supernatant was presorbed with Protein G Sepharose or IgM Sepharose (Zymed) for 30 min at 4°C, then recovered and incubated for 2 hr at 4°C with either 10 μl of purified SC1 antibody (gift from Dr. H. Sage) or 50 μl of RGRS1-coupled IgM Sepharose, respectively. To the brain extract containing SC1 antibody, 50 μl of Protein G Sepharose was added and incubated overnight at 4°C, while the RGRS1-IgM IP was allowed to continue incubation overnight at 4°C. Both IPs were washed 4 times, 10 min each, at 4°C with PBS containing 10% glycerol, then analyzed for protein binding by SDS-PAGE followed by Western blotting with the SC1 antibody.

Immunohistochemistry

The following primary antibodies were used: RGRS1 mAbs, Tuj-1 (Covance), rat anti-SC1 antibody (gift from Dr. H. Sage), Rat-401 and RC2 (Iowa Hybridoma Bank), polyclonal anti-GFAP (Dako), and polyclonal anti-BLBP (Dr. N. Heintz, Rockefeller University). GFP expression in hGFAP-GFP mouse cortical cells was detected with rabbit anti-GFP antibodies (Abcam). RGRS1 and RC2 are of IgM isotype, whereas others are of IgG isotype. Cryosections of embryonic cerebral cortices and primary embryonic cortical cells were immunolabeled as described earlier and immunoreactivity was detected with appropriate Cy-3-, AMCA-, or FITC-conjugated secondary antibodies.

Analysis of SC1 Expression on Neuronal Adhesion In Vitro

5 μg of pSC1-IRES-EGFP or pIRES-EGFP was transfected into Cos-7 or C6R cells using the lipofectamine PLUS system (Invitrogen). Twenty-four hours later, E16 mouse cortices were dissociated, filtered through a cell strainer (Falcon), and added to transfected Cos-7 or C6R cells at a concentration of 100,000 cells/35 mm dish. After 24 hr of incubation, cells were fixed and processed for immunocytochemistry with Tuj-1 antibodies. The total number of EGFP-positive cells and the number of EGFP-positive cells that made contact with or attached to Tuj-1-positive neurons were quantified. In some experiments, E16 dissociated cortical cells were transfected with SC1-EGFP or EGFP soon after dissociation, cultured for 72 hr, and labeled with anti-GFAP and Tuj-1 antibodies, and the extent of contact between transfected astroglia (i.e., GFP- and anti-GFAP-positive) and Tuj-1-positive neurons were quantified.

Slice Adhesion Assays

We modified a method developed by Emerling and Lander (1994) to assess the effect of SC1 on adhesion of embryonic cortical cells to cortical slices in culture. 300 μm slices of E16 mouse cortices were placed on nucleopore membrane filters (Whatman) and incubated at 37°C in either RGRS1 antibodies, control antibodies, purified recombinant SC1 protein (30 ng/ml; gift from Dr. E. Sage, Hope

Heart Inst., WA; generated in a baculovirus expression system; Bradshaw et al., 2000; Soderling et al., 1997), or RGRS1 antibody presorbed with purified SC1 protein (30 ng/ml, mixed and incubated together overnight prior to addition to culture). In parallel, dissociated E14 cortical cells were labeled with CMTMR [(5-(and-6)-(((4-chloromethyl)benzoyl) amino) tetramethyl rhodamine); Molecular Probes] cell tracker dye for 15 min, and washed 4 times with MEM/5% horse serum. Labeled cells were then seeded onto cortical slices (25,000 cells per well) and incubated for 24 hr at 37°C. Images of CMTMR-labeled cortical cells attached to cortical slices were acquired and analyzed with Metamorph software (Universal Imaging Inc.).

Electroporation and Slice Cultures for Migration Assays

Lateral ventricles of E15.5 mouse embryos were injected with 1.5 μl of a plasmid mixture, containing 4 μg/μl DNA (pSC1-IRES-EGFP or pIRES-EGFP) diluted 1:1 with mouse neuron nucleofector solution (Amaxa biosystems)/0.001% fast green, using a Parker Hannifin Picospritzer II. Immediately after injection, heads were subjected to electroporation (BTX/Genetronics) under the following conditions: LV mode, 70 Volts, 100 ms pulse length, 100 ms pulse interval, 8 pulses, unipolar [polarity]. Following electroporation, cortices were removed from the embryos, coronally sectioned (150 μm), mounted on nucleopore membrane filters, and cultured in MEM/10% FBS at 37°C/5% CO_2 for 24 hr. 10 μm BrdU was then added to the cultures and slices were maintained for an additional 24 hr, prior to labeling with anti-BrdU antibodies. In each slice, the extent of migration of GFP-positive neurons into the cerebral wall away from the ventricular zone was measured as follows: (width of the cerebral wall − distance of GFP-positive cell from pia)/width of the cerebral wall.

For live imaging of SC1-GFP- or control GFP-expressing neurons, GFP-labeled neurons in the intermediate zone of the slices were repeatedly imaged using a Zeiss inverted microscope (attached to a confocal laser scanning system and a live cell incubation chamber) for 2–3 hr. The rate of migration of the monitored cells was measured using LSM5 Pascal program (Zeiss).

SC1 Mutant Mice and BrdU Birthdating Studies

Generation and characterization of targeted mutation in mouse SC1 was described in McKinnon et al. (2000). For BrdU birthdating analysis, pregnant mice were injected intraperitoneally with BrdU (10 mg/kg body weight, Sigma) on embryonic day 16. Wild-type and mutant mice were removed 6 weeks after birth and processed for Nissl or BrdU labeling as described earlier (Anton et al., 1999). The comparisons between sections from different embryos were obtained from identical cortical regions corresponding to anterior occipital areas. For each section, the distance of BrdU-labeled neurons at the top and bottom of the layers to the pial surface and the corresponding widths of the cerebral cortex was measured. The width of the distribution of BrdU-labeled neurons in each section was normalized to the width of the cortex. The position of BrdU+ cells within the cerebral wall also was quantified.

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References

Anderson, S.A., Eisenstat, D.D., Shi, L., and Rubenstein, J.L. (1997). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. Science *278*, 474–476.

Ang, E.S., Haydar, T.F., Gluncic, V., and Rakic, P. (2003). Four-dimensional migratory coordinates of GABAergic interneurons in the developing mouse cortex. J. Neurosci. *23*, 5805–5815.

Angevine, J.B., and Sidman, R.L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. Nature *192*, 766–768.

Anton, E.S., Sandrock, A., and Matthew, W.D. (1994). Merosin promotes neurite growth and Schwann cell migration *in vitro* and nerve regeneration *in vivo*: evidence using an antibody to merosin, ARM-1. Dev. Biol. *164*, 133–146.

Anton, E.S., Marchionni, M., Lee, K.-F., and Rakic, P. (1997). Role of GGF/Neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. Development *124*, 3501–3510.

Anton, E.S., Kreidberg, J., and Rakic, P. (1999). Distinct functions of α_3 and α_v integrin receptors in neuronal migration and laminar organization of the cerebral cortex. Neuron *22*, 277–289.

Bradshaw, A.D., Bassuk, J.A., Francki, A., and Sage, E.H. (2000). Expression and purification of recombinant human SPARC produced by baculovirus. Mol. Cell Biol. Res. Commun. *3*, 345–351.

Brekken, R.A., and Sage, E.H. (2000). SPARC, a matricellular protein: at the crossroads of cell-matrix communication. Matrix Biol. *19*, 569–580.

Costell, M., Gustafsson, E., Aszodi, A., Morgelin, M., Bloch, W., Hunziker, E., Addicks, K., Timpl, R., and Fassler, R. (1999). Perlecan maintains the integrity of cartilage and some basement membranes. J. Cell Biol. *147*, 1109–1122.

D'Arcangelo, G., Miao, G.G., Chen, S.-C., Soares, H.D., Morgan, J.I., and Curren, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant *reeler*. Nature *374*, 719–723.

de Carlos, J.A., Lopez-Mascaraque, L., and Valverde, F. (1996). Dynamics of cell migration from the lateral ganglionic eminence in the rat. J. Neurosci. *16*, 6146–6156.

Emerling, D.E., and Lander, A.D. (1994). Laminar specific attachment and neurite outgrowth of thalamic neurons on cultured slices of developing cerebral neocortex. Development *120*, 2811–2822.

Frantz, G.D., and McConnell, S.K. (1996). Restriction of late cerebral cortical progenitors to an upperlayer fate. Neuron *17*, 55–61.

Friedlander, D.R., Brittis, P.A., Sakurai, T., Shif, B., Wirchansky, W., Fishell, G., and Grumet, M. (1998). Generation of a radial-like glial cell line. J. Neurobiol. *37*, 291–304.

Gilles, C., Bassuk, J.A., Pulyaeva, H., Sage, E.H., Foidart, J.M., and Thompson, E.W. (1998). SPARC/osteonectin induces MMP2 activation in human breast cancer cell lines. Cancer Res. *58*, 5529–5536.

Girard, J.P., and Springer, T.A. (1996). Modulation of endothelial cell adhesion by hevin, an acidic protein associated with high endothelial venules. J. Biol. Chem. *271*, 4511–4517.

Halfter, W., Dong, S., Yip, Y.P., Willem, M., and Mayer, U. (2002). A critical function of the pial basement membrane in cortical histogenesis. J. Neurosci. *22*, 6029–6040.

Hambrock, H.O., Nitsche, D., Hansen, U., Bruckner, P., Paulsson, M., Maurer, P., and Hartmann, U. (2003). SC1/Hevin. An extracellular Ca^{2+} modulated protein that binds collagen. J. Biol. Chem. *278*, 11351–11358.

Hartfuss, E., Forster, E., Bock, H.H., Hack, M.A., Leprince, P., Luque, J.M., Herz, J., Frotscher, M., and Gotz, M. (2003). Reelin signaling directly affects radial glia morphology and biochemical maturation. Development *130*, 4597–4609.

Hatten, M.E., and Mason, C.A. (1990). Mechanisms of glial-guided migration in vitro and in vivo. Experientia *46*, 907–916.

Hu, H. (1999). Chemorepulsion of neuronal migration by Slit2 in the developing mammalian forebrain. Neuron *23*, 703–711.

Johnston, I.G., Paladino, T., Gurd, J.W., and Brown, I.R. (1990). Molecular cloning of SC1: a putative brain ECM glycoprotein showing partial similarity to osteonectin/BM40/SPARC. Neuron *4*, 165–176.

Komuro, H., and Rakic, P. (1996). Intracellular Ca2+ fluctuations modulate the rate of neuronal migration. Neuron *17*, 275–285.

Kriegstein, A.R., and Gotz, M. (2003). Radial glia diversity: a matter of cell fate. Glia *43*, 37–43.

Marin, O., and Rubenstein, J.L.R. (2003). Cell migration in the forebrain. Annu. Rev. Neurosci. *26*, 441–483.

Maurer, P., Hohenadl, C., Hohenester, E., Gohring, W., Timpl, R., and Engel, J. (1995). The C-terminal portion of BM-40 (SPARC/osteonectin) is an autonomously folding and crystallisable domain that binds calcium and collagen IV. J. Mol. Biol. *253*, 347–357.

McConnell, S.K. (1990). The specification of neuronal identity in the mammalian cerebral cortex. Experientia *46*, 922–929.

McConnell, S.K., and Kaznowski, C.E. (1991). Cell cycle dependence of laminar determination in developing neocortex. Science *254*, 282–285.

McKinnon, P.J., and Margolskee, R.F. (1996). SC1: a marker for astrocytes in the adult rodent brain is upregulated during reactive astrocytosis. Brain Res. *709*, 27–36.

McKinnon, P.J., Kapsetaki, M., and Margolskee, R.F. (1996). The exon structure of the mouse Sc1 gene is very similar to the mouse Sparc gene. Genome Res. *6*, 1077–1083.

McKinnon, P.J., McLaughlin, S.K., Kapsetaki, M., and Margolskee, R.F. (2000). Extracellular matrix associated protein SC1 Is not essential for mouse development. Mol. Cell. Biol. *20*, 656–660.

Mendis, D.B., Shahin, S., Gurd, J.W., and Brown, I.R. (1996). SC1, a SPARC-related glycoprotein, exhibits features of an ECM component in the developing and adult brain. Brain Res. *713*, 53–63.

Miner, J.H., Cunningham, J., and Sanes, J.R. (1998). Roles for laminin in embryogenesis: exencephaly, syndactyly, and placentopathy in mice lacking the laminin alpha5 chain. J. Cell Biol. *143*, 1713–1723.

Miyata, T., Kawaguchi, A., Okano, H., and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron *31*, 727–741.

Mothe, A.J., and Brown, I.R. (2001). Differential mRNA expression of the related glycoproteins SC1 and SPARC in the rat embryonic nervous system and skeletal structure. Brain Res. *892*, 27–41.

Muneoka, K., Wanek, N., Trevino, C., and Bryant, S.V. (1990). Exo-utero surgery. In Post-Implantation Mammalian Embryo A. Coops and D. Cockroft, eds. (Oxford: IRL Press), pp. 41–59.

Nadarajah, B., Brunstrom, J.E., Grutzendler, J., Wong, R.O., and Pearlman, A.L. (2001). Two modes of radial migration in early development of the cerebral cortex. Nat. Neurosci. *4*, 143–150.

Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S., and Kriegstein, A.R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. Nature *409*, 714–720.

Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Selke, M., Ikenaka, K., Yamamoto, H., and Mikoshiba, K. (1995). The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron *14*, 899–912.

Olson, E.C., and Walsh, C.A. (2002). Smooth, rough and upside-down neocortical development. Curr. Opin. Gen. Dev. *12*, 320–327.

O'Rourke, N.A., Dailey, M.E., Smith, S.J., and McConnell, S.M. (1992). Diverse migratory pathways in the developing cerebral cortex. Science *258*, 299–302.

Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. J. Comp. Neurol. *145*, 61–84.

Rakic, P. (1974). Neurons in the monkey visual cortex: systematic relation between time of origin and eventual disposition. Science *83*, 425–427.

Rakic, P. (1988). Defects of neuronal migration and pathogenesis of cortical malformations. Prog. Brain Res. *73*, 15–37.

Rakic, P. (2003). Elusive radial glia: historical and evolutionary perspective. Glia *43*, 19–32.

Rakic, P., and Caviness, V.S. (1995). Cortical development: view from neurological mutants two decades later. Neuron *14*, 1101–1104.

Sasaki, T., Hohenester, E., Gohring, W., and Timpl, R. (1998). Crystal structure and mapping by site-directed mutagenesis of the collagen-binding epitope of an activated form of BM-40/SPARC/osteonectin. EMBO J. *17*, 1625–1634.

Sidman, R.L., and Rakic, P. (1973). Neuronal migration with special reference to the developing human brain: a review. Brain Res. *62*, 1–35.

Soderling, J.A., Reed, M.J., Corsa, A., and Sage, E.H. (1997). Cloning and expression of murine SC1, a gene product homologous to SPARC. J. Histochem. Cytochem. *45*, 823–835.

Takahashi, T., Misson, J.-P., and Caviness, V.S. (1990). Glial process elongation and branching in the developing murine neocortex: a qualitative and quantitative immunohistochemical analysis. J. Comp. Neurol. *302*, 15–28.

Valcanis, H., and Tan, S.S. (2003). Layer specification of transplanted interneurons in developing mouse neocortex. J. Neurosci. *23*, 5113–5122.

Wu, W., Wong, K., Chen, J., Jiang, Z., Dupuis, S., Wu, J.Y., and Rao, Y. (1999). Directional guidance of neuronal migration in the olfactory system by the protein Slit. Nature *400*, 331–336.

Yan, Q., and Sage, E.H. (1999). SPARC, a matricellular glycoprotein with important biological functions. J. Histochem. Cytochem. *47*, 1495–1506.

Zheng, C., Heintz, N., and Hatten, M.E. (1996). CNS gene encoding astrotactin, which supports neuronal migration along glial fibers. Science *272*, 417–419.