Sonic hedgehog restricts adhesion and migration of neural crest cells independently of the Patched-Smoothened-Gli signaling pathway

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In the vertebrate embryo, neural cell types are organized spatially along the dorsoventral axis of the neural tube and differ by expression of cell-intrinsic determinants and by their adhesive and locomotory properties. Thus, dorsally, neural crest cells (NCC) show a strong propensity to disperse and migrate, whereas cells situated ventrally are highly cohesive and poorly motile. Members of the bone morphogenetic proteins have been shown to exert a dual role in the specification of dorsal neuroepithelial cells and in the dispersion of NCCs. To test whether Sonic hedgehog (Shh), another signaling molecule involved in the patterning of the ventral neural tube, might also contribute to the control of the adhesive and migratory potential of neuroepithelial cells, we analyzed the effect of ectopic Shh on NCC dispersion from neural tube explants cultured *in vitro*. The addition of Shh to the migration substrate of NCC caused inhibition of their dispersion. The effect of Shh on cell migration was reversible and was not accounted for by alterations of the specification, delamination, proliferation, and survival of NCCs but could be essentially attributed to a decreased cell-substrate adhesion mediated by integrins. In addition, Shh activity on cell migration was mediated by a specific N-terminal region of the molecule and was independent from the signaling cascade elicited by the Patched-Smoothened receptor and involving the Gli transcription factors. Our study therefore reveals an unanticipated role for Shh in regulating adhesion and migration of neuroepithelial cells that is discernable from its inductive, mitogenic, and trophic functions.

In higher vertebrate embryos, the generation of neural cell types along the dorsoventral axis of the spinal cord requires the antagonistic activities of inductive signals emanating from adjacent tissues that are capable of patterning cell fates at a distance (1–4). Thus, Sonic hedgehog (Shh), a member of the hedgehog (hh) family of signaling molecules expressed by the notochord and floor plate, acts in a graded manner to direct the differentiation of motoneurons and adjacent interneurons in the ventral neural tube. Conversely, the bone morphogenetic proteins, BMP4 and BMP7, which are secreted by the ectoderm and the roof plate, drive sequentially the specification of neural crest cells (NCCs), roof plate cells, and sensory interneurons in the dorsal neural tube.

The inductive signals implicated in the patterning of the nervous system act primarily on the expression of cell-intrinsic determinants, typically transcription factors. However, cell types of the neural tube also exhibit specific adhesive and migratory properties. For example, cells in the floor plate are poorly motile but instead are organized in a tight epithelium-like structure. Other cells, like motoneuron progenitors, lose contact with the luminal side of the neuroepithelium and migrate toward the periphery at the basal side. Lastly, unlike all other neuroepithelial cells, NCCs are a transient resident of the neural tube as they delaminate from its dorsal side soon after their specification and acquire locomotory properties, allowing them to disperse through the adjacent tissues (5–7). As exemplified for NCCs, the repertoire of integrins and cadherins, two major families of adhesion molecules, is profoundly remodeled during specification and segregation of cell lineages in the spinal cord. At the time of invagination and folding of the neural plate, E-cadherin disappears from the neural folds to become restricted to the superficial ectoderm in contrast to N-cadherin, whose expression domain expands dorsally up to the boundary between the ectoderm and the neural tube (8, 9). Later, NCC delamination from the neural tube coincides with the extinction of both N-cadherin and cadherin-6B in NCCs, which instead express cadherin-7 (10). Concomitantly, NCCs express the $\alpha 4\beta 1$ integrin and acquire the ability to respond to fibronectin (FN), an extracellular matrix molecule highly suitable for migration (11). Functional studies revealed that inhibition of integrin function or misexpression of cadherins both results in the accumulation of NCCs in the dorsal neural tube, indicating that the concerted shift of adhesion molecules is essential for cell dispersion (10, 12, 13).

The factors orchestrating changes in adhesive and migratory events during the patterning of the neural tube remain ill-defined. In the avian embryo it has been shown that the coordinated activities of BMPs and their specific antagonists regulate NCC dispersion by modifying expression and activity of integrins and cadherins (14, 15). Furthermore, when challenged with BMPs, ventral neural tubes can generate cells that display characteristics of migrating NCCs (1, 16), indicating all neuroepithelial cells retain at least transiently the potential to migrate and that cells situated in the ventral neural tube are prevented to do so, presumably because of the activity of the inhibitors. Given the antagonistic activities of BMPs and Shh for the generation of dorsal and ventral neural tube cells, Shh is a candidate molecule for restricting ventral cell dispersion. In the present study, to test this hypothesis, we analyzed the effect of Shh on NCC dispersion from neural tube explants cultured *in vitro*. We found that Shh blocked NCC adhesion and migration and it affected integrin function by a mechanism which is independent from the Patched-Smoothened-Gli signaling pathway.

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Materials and Methods

Soluble Proteins, Abs, and cDNA Probes. Production and purification of soluble human Shh, the Shh(PolyQ) variant with the N-terminal residues 32–38 (KRRHPKK) mutated to QQQH-

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Abbreviations: FN, fibronectin; LN1, laminin-1; NCC, neural crest cell; Shh, Sonic hedgehog; VN, vitronectin; BMP, bone morphogenetic protein; HNK1, human natural killer-1.

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PQQ, and the truncated Shh(N-9/C-3) variant were described elsewhere (17). The mAb ASC1 has been raised to a human Shh-Fc fusion protein as described (17). The mAb 5E1 to chicken Shh was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Polyclonal Abs and mAb ES66–8 to the chick integrin β1 chain were obtained from K. Yamada (National Institutes of Health, Bethesda), Abs to the chick integrin α1 chain were obtained from M. Paulsson (University of Cologne, Cologne, Germany), and the mAb Chav1 to the chick integrin αv chain were obtained from L. Reichardt (University of California, San Francisco). cDNAs for *Ncad*, *cad6B*, and *cad7* were provided by M. Takeichi (Kyoto University, Kyoto, Japan), *Shh* was provided by C. Tabin (Harvard Medical School, Boston), *BMP4* was provided by P. Brickell (Institute of Child Health, London), *Noggin* was provided by L. Niswander (Memorial Sloan Kettering Cancer Center, New York), *Bmp7* was provided by A.-H. Monsoro-Burq (Institut d'Embryologie, Nogent-sur-Marne, France), *Slug* was provided by A. Nieto (Instituto Cajal, Madrid), *RhoB* was provided by I. de Curtis (DIBIT, Milan, Italy), and *Pax3* was provided by P. Gruss (Max Planck Institute, Goettingen, Germany).

Cell Cultures and Assays for Cellular Adhesion and Migration. Cultures of truncal NCCs were generated from quail or chick embryos at the 20–25-somite stage as described (18). Assays for cellular adhesion and migration were performed as described (18, 19) in culture dishes coated with bovine plasma FN (Sigma), mouse laminin-1 (LN1; Sigma), or bovine vitronectin (VN; purified as described in ref. 20). For adhesion assays in the presence of metabolic agents, cells were preincubated for 15 min with actinomycin-D (Sigma), cycloheximide (Sigma), forskolin (Sigma), or cyclopamine (a gift from W. Gaffield, Western Regional Research Center, Albany, CA) before plating.

***In Situ* Hybridization and Immunolabeling.** *In situ* hybridizations were carried out as described (21) by using alkaline phosphatase-conjugated anti-digoxigenin Ab and were revealed with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, which yields a purple precipitate. *In situ* hybridizations were generally followed by immunoperoxidase staining for simultaneous detection of NCCs by human natural killer-1 (HNK1). Peroxidase activity was revealed by using diaminobenzidine as a chromogen, producing a brown precipitate. To study the expression pattern of integrins in cell cultures, aliquots of neural tube cells were incubated at 4°C with the anti-integrin Abs and the mAb HNK1 and subjected to immunofluorescent staining by using fluorescein- or phycoerythrin-conjugated anti-mouse or anti-rabbit Abs. Cells were analyzed by flow cytometry, using a Coulter Elite cell sorter.

Determination of Cell Proliferation and Cell Death. The cell cycle was analyzed by flow cytometry after DNA staining by using propidium iodide. Neural tube cells were collected and subjected to HNK1 staining by using phycoerythrin-conjugated anti-mouse Ab, fixed in 70% ethanol, and treated with RNaseA at 1 mg/ml before propidium iodide was added for flow cytometry analysis. Cell proliferation was measured by immunohistochemical detection of BrdUrd incorporation on neural tube cultures, using the labeling and detection kit from Roche. Apoptotic cells were detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) on neural tube cultures, using the *in situ* cell death detection kit from Roche.

Results

Shh Prevents NCC Migration *in Vitro*. To investigate whether Shh might regulate adhesion and migration of neuroepithelial cells, we analyzed its effect on NCC dispersion out of truncal neural tube explants cultured on FN substrates in the presence of the biologically active N-terminal fragment of Shh. Because Shh can diffuse and act at some distance or remain associated with the cell surface through coupling to cholesterol and binding to proteoglycans or extracellular matrix molecules (22–24), we tested its effect under either an immobilized form adsorbed onto the dish or in solution. Shh was applied in solution at 0.1–5 μg/ml or coated onto the dish at 1–20 μg/ml, given that only 0.1–2 μg/ml remained effectively adsorbed onto the plastic (unpublished observations).

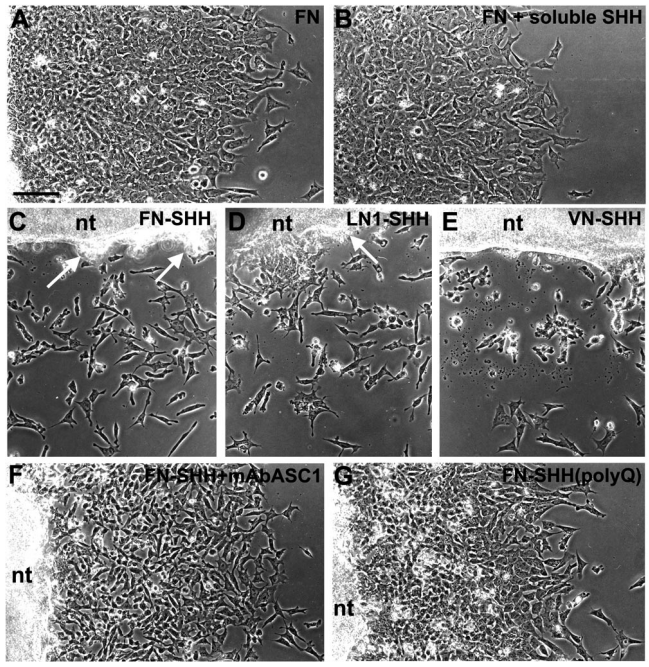

Fig. 1. *In vitro* NCC migration is impaired by Shh. (*A–E*) NCC outgrowths after 18 h on FN at 2 μg/ml (*A*); in the presence of Shh added in the culture medium at 5 μg/ml (B); and in the presence of Shh adsorbed at 20 μg/ml on the substrate with FN at 2 μg/ml (*C*), LN1 at 20 μg/ml (*C*), or VN at 5 μg/ml (*D*). Cell migration is severely reduced when Shh is adsorbed on the substrate, and most NCCs remain as clusters on top of the neural tube (arrows). (*F* and *G*) NCC migration after treatment with Shh at 20 μg/ml in the presence mAb ASC1 to Shh added at 150 μg/ml in the culture medium (*F*) or with the biologically inactive Shh(polyQ) mutant adsorbed on the substrate at 20 μg/ml (*G*). nt, neural tube. (Bar = 100 μm.)

On FN substrates in the absence of Shh (Fig. 1*A*), NCCs organized into a dense outgrowth surrounding the neural tube explant after 18 h in culture and exhibited their typical stellate morphology. When Shh was adsorbed onto the substrate at 10–20 μg/ml with FN, cell dispersion was strongly impaired. Only some cells spread poorly were scattered on the substrate and most of them remained as clusters on top of the neural tube (Fig. 1*C*). Quantitation of the number of cells attached to the substrate revealed that the number of migrating cells was dramatically reduced (Table 1). In contrast, when Shh was applied in solution, it was considerably less potent even at high concentrations, and NCCs migrated almost normally (Fig. 1*B*), indicating that Shh had to be presented under an immobilized form to be effective. In all of the subsequent experiments, Shh was adsorbed onto the substrate at 20 μg/ml. The severe reduction in cell migration could not be attributed to Shh competition with FN for adsorption to the dish, as the estimation of the relative amount of adsorbed FN showed a reduction by only 10–30% at the highest doses of Shh. Conversely, increasing FN concentration up to 100 μg/ml did not have a significant affect on Shh coating to the dish (data not shown). Shh also abolished migration over LN1 or VN, two other matrix mole-

Table 1. Effect of Shh on the no. of NCCs migrating out of the neural tube

Treatment	Average no. of migrating NCCs per neural tube explant ± SD	% of control
FN, 2 μg/ml	2555 ± 665	—
FN + Shh, 5 μg/ml	1390 ± 339	54
FN + Shh, 10 μg/ml	722 ± 62	28
FN + Shh, 20 μg/ml	700 ± 191	27
FN + Shh(polyQ), 20 μg/ml	2146 ± 84	84
FN + Shh(N-9/C-3), 20 μg/ml	2363 ± 713	93
FN + Shh (20 μg/ml) + mAb ASC1 (100 μg/ml)	1946 ± 394	76
FN + Shh (20 μg/ml) + mAb 5E1 (100 μg/ml)	114 ± 50	5

Neural tube explants were grown on FN in the presence of Shh at different concentrations or of mutated constructs of Shh. Abs to Shh were applied to the culture before the neural tubes were deposited into the dish. After 18 h in culture, the neural tubes and associated NCC aggregates were removed from the dish with needles, and the remaining NCC population that migrated over the substrate was collected with EDTA treatment and counted under a microscope.

cules known to support NCC migration (19, 25), suggesting that its effect did not result from a specific association with the FN molecule (Fig. 1 *D* and *E*).

To assess further the specificity of Shh effect on NCC dispersion, mAbs to Shh were applied to the cultures to inhibit its function. As shown in Fig. 1*F*, NCC migration was restored in the presence of the mAb ASC1, which was selected for its ability to block cell response to Shh. Likewise, when the Shh(polyQ) mutant, a construct of Shh lacking biological activity (17), was substituted for wild-type Shh on the substrate, NCC migration occurred almost normally (Fig. 1*G*). Estimation of the number of migrating cells confirmed that with the mAb ASC1 and the Shh(polyQ) mutant migration was restored to normal levels (Table 1). We next studied whether the effect of Shh could be reverted by transferring neural tubes that were previously confronted with Shh to Shh-free substrates. Within less than 1 h, NCC dissociated from the clusters attached to the neural tube, spread on the substrate, and resumed migration; by 4–8 h, they formed a large outgrowth virtually indistinguishable from those obtained under normal conditions (data not shown).

Because Shh acts primarily as a specification, mitogenic, and trophic factor in the nervous system (26–28), we examined whether the absence of NCC migration on Shh-containing substrates resulted from alterations in cell fate, proliferation, or survival. The expression patterns of *Shh* and *Pax3*, which mark the dorsal and ventral cell populations of the neural tube, respectively, were neither expanded nor reduced in the presence of exogenous Shh, providing evidence that the dorsoventral polarity of the neural tube was apparently not perturbed in our experiments (data not shown). In addition, cells in clusters on top of the neural tube and those integrated in its dorsal third expressed both NCC markers *Slug* and HNK1, indicating that NCCs were able to pursue their differentiation process *in situ* even though they failed to migrate (Fig. 2*A* and *B*). Finally, quantitative analyses showed that neither the numbers of neural tube and NCCs nor the proportions of proliferating and apoptotic cells in the neural tube and NCC populations were significantly altered after exposure to Shh (Table 2 and data not shown). These results clearly demonstrate that neither a deficit in the production of NCCs nor changes in their proliferation and survival could account for the Shh inhibition of cell dispersion.

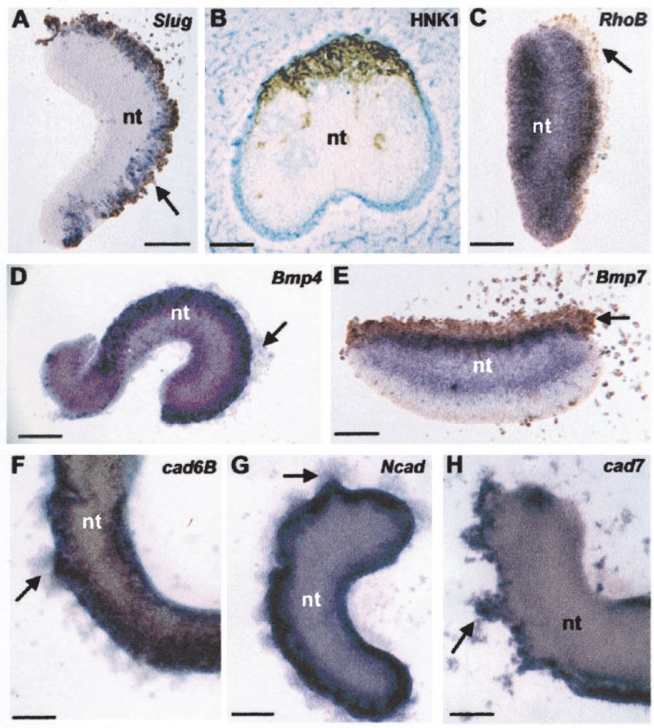

Fig. 2. Shh does not affect NCC specification and delamination. (*A*) Simultaneous detection of *Slug* mRNA and HNK1 epitope on neural tube explants confronted with Shh at 20 μg/ml shows NCCs packed in clusters along the dorsal margin of the neural tube. (*B*) HNK1 labeling of a transverse section through a Shh-treated neural tube explant reveals NCCs inside the dorsal neural tube itself. (*C–H*) Expression patterns of *RhoB* (*C*), *Bmp4* (*D*), *Bmp7* (*E*), *cad6B* (*F*), *Ncad* (*G*), and *cad 7* (*H*) in neural tubes exposed to Shh at 20 μg/ml, showing that NCC aggregates express *cad7* but not *RhoB*, *Ncad*, or *cad6B* like their normal counterparts, whereas *Bmp4* and *Bmp7* are found at normal levels in the dorsal side of the neural tube. In *C* and *E*, explants were immunolabeled for HNK1. Arrows point at NCC clusters. nt, neural tube. [Bars = 100 μm (for *A* and *C–H*) and 50 μm (for *B*).]

Shh Does Not Affect NCC Delamination. A possible explanation for the failure of NCCs to disperse from the neural tube is that Shh perturbed the cascade of events leading to their delamination. Recent evidence suggests that during delamination, NCCs undergo an epithelium to mesenchyme transition under the control of BMP signals regulating expression of cadherins and RhoB, a member of the Rho family of GTPases (10, 15, 29). Analyses of the expression patterns of *Bmp4*, *Bmp7*, and *Noggin* in neural tubes confronted with Shh did not reveal striking changes in the level of their expression or in their spatial distribution as compared with controls (Fig. 2 *D* and *E* and data not shown). In addition, *RhoB* transcripts were found in the neural tube and not in HNK1-positive cells (Fig. 2*C*), indicating that in NCCs confronted with Shh, *RhoB* was down-regulated after delamination as under normal conditions. Finally, NCCs, whether they were dispersed on the substrate or aggregated on the neural tube, expressed high levels of *cad7* messages and little or no *cad6B* and *Ncad* (Fig. 2 *F–H*). These results then rule out the possible implication of a mechanism perturbing BMP-dependent signals that would affect NCC delamination.

Shh Affects Integrin-Mediated Adhesion and Migration of NCCs. Integrins play a key role in NCC adhesion and migration. To determine whether the effect of Shh on NCC dispersion could be mediated by changes in the repertoire and function of integrins, we analyzed by flow cytometry and immunofluorescence the patterns of expression of the β1, α1, αv, and α4 chains

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Table 2. Effect of Shh on NCC proliferation and survival

Parameters	FN without Shh	FN + Shh
Total number of cells per explant (mean ± SD)	13,129 ± 3,967	10,741 ± 3,206
% of HNK1-positive cells per explant	10.5 ± 2.0	9.2 ± 3.0
% of cells (neural tube + NCC) in the S phase	25.7 ± 1.6	24.5 ± 1.7
% of HNK1-positive cells in the S phase	24.4 ± 1.8	21.3 ± 2.0
% of apoptotic cells among the NCC population	2.3	4.5

To determine the total number of cells and the proportion of NCCs after Shh treatment, neural tube explants cultured on FN in the presence of Shh at 20 μg/ml were dissociated into a single-cell suspension. Cells were collected, counted, and immunolabeled for the HNK1 epitope, and the proportion of HNK1-positive cells was estimated by flow cytometry. The proportions of NCCs in the S phase or of apoptotic cells were measured by flow cytometry after DNA labeling with propidium iodide and HNK1 labeling.

known to be essential for NCC adhesion and migration on FN, LN1, and VN (19, 25, 30). We also measured direct adhesion of NCCs to the substrate. Exposure to Shh apparently did not modify the surface expression of integrins in NCCs (Fig. 3*A* and not shown). However, cell adhesion to FN (Fig. 3*B*), LN1, or VN (data not shown) was severely reduced at low doses of substrate molecules but was normal at high concentrations, suggesting that Shh inhibited integrin activity merely by acting as a competitor for ligand binding. The Shh(polyQ) mutant, in contrast, showed only a minimal effect on cell adhesion at all concentrations of FN (Fig. 3*B*), LN1, or VN (data not shown). Analysis of the kinetics of cell adhesion showed that Shh effect was immediate and maximal for approximately 1 h. Significantly, the profile of the dose-response curve of the effect of Shh on cell adhesion paralleled the migration curve (Fig. 3*B*), thereby reinforcing the idea that the deficit in cell migration induced by Shh merely resulted from the inhibition of cell adhesion.

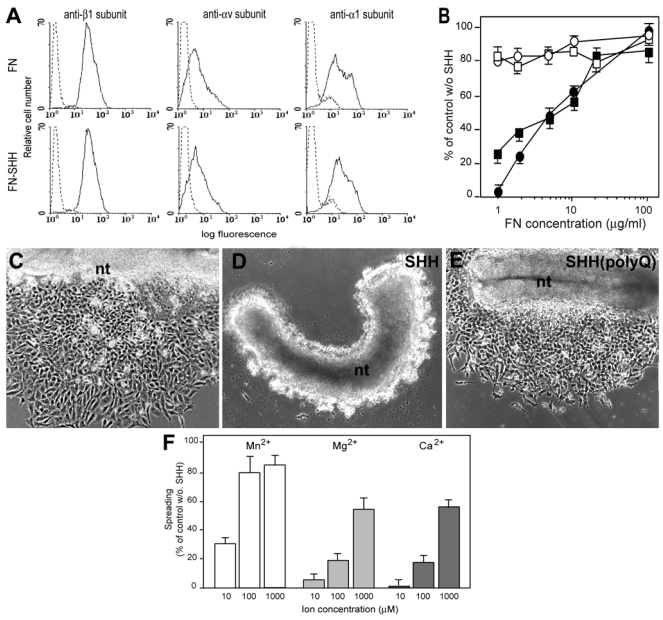

Fig. 3. Shh affects integrin-mediated adhesion and migration of NCCs. (*A*) Flow cytometry analysis of integrins β1, α1, and αv subunit expression in neural tube and NCCs reveals no major differences between cells collected from explants cultured during 18 h on FN in the absence (*Top*) or presence (*Bottom*) of Shh at 20 μg/ml. (*B*) NCC spreading (squares) and migration (circles) on FN at different concentrations from 1 μg/ml to 100 μg/ml in the presence of Shh (filled symbols) or of the Shh(polyQ) mutant (open symbols) at 20 μg/ml. Spreading was measured by using NCCs that have been collected from explants previously cultured in the absence of Shh. (*C–E*) NCC outgrowths after 18 h on the mAb ES66–8 anti-β1 integrin used as a substrate at 100 μg/ml in the absence of Shh (*C*), or in the presence of Shh (*D*) or of the Shh(PolyQ) mutant (*E*), each adsorbed at 20 μg/ml on the substrate. (*F*) Effects of Mn^{2+}, Ca^{2+}, and Mg^{2+} on NCC spreading on FN in the presence of Shh at 20 μg/ml. Mn^{2+} can totally abrogate the effect of Shh on cell spreading, whereas Ca^{2+} and Mg^{2+} are much less effective even at high concentrations. nt, neural tube. [Bar = 100 μm.]

To demonstrate further that Shh affected integrin-mediated adhesion, we tested its effect on NCC migration over substrates in which FN was replaced by Abs to the integrin β1 chain. When used as a substrate, such Abs efficiently promote NCC migration in a manner that is believed to rely exclusively on integrins (31). As shown on Fig. 3*C*, NCCs displayed essentially the same morphology and covered the same distance on the mAb ES66–8 anti-β1 integrin as on FN. In the presence of Shh but not of the Shh(polyQ) mutant, cell migration over the Ab was entirely blocked, and almost all NCCs appeared as clusters along the dorsal margin of the neural tube (Fig. 3 *D* and *E*). These results argue in favor of a direct effect of Shh on integrin function in NCCs.

Conformational changes in integrins have been suggested to underlie the modulation of their binding to extracellular matrix ligands and can be artificially induced by specific cations or Abs to the β chain (32). To test whether Shh could inhibit NCC adhesion by converting integrins into an inactive conformation, we sought to antagonize its effect in adhesion assays by exposing cells to Mn^{2+}, known to induce highly active conformation of integrins. Fig. 3*F* shows that Mn^{2+}, but not Mg^{2+} or Ca^{2+}, added to the culture medium was able to counteract almost completely the effect of Shh on NCC adhesion, therefore suggesting that Shh could act by inducing a conformational shift of integrins toward an inactive state.

Shh Effect on Cell Adhesion and Migration Is Not Mediated by the Patched-Smoothened-Gli Signaling Pathway. To decipher in molecular terms the mechanism by which Shh controls integrin function in NCCs, we sought to characterize, using a variety of mAbs to Shh and mutated constructs of the molecule, which Shh sequences were implicated in this effect and to analyze whether they differ from those required for motoneuron specification in neural tube explants (17). The positions of the mutated sites and of the mAb-binding epitopes are illustrated in Fig. 4*A* and their functional properties are summarized in Table 3. As mentioned previously, the Shh(polyQ) mutant, which is not able to promote motoneuron differentiation and does not bind the Patched receptor with a significant affinity, was unable to bock NCC adhesion and migration. Likewise, the Shh(N-9/C-3) mutant also failed to inhibit adhesion and migration (Fig. 4*B*, Table 1) although it retains the ability to bind the Patched receptor and to promote motoneuron differentiation with the same efficacy as Shh (17). On the other hand, in striking contrast to the mAb ASC1, the mAb 5E1, originally developed for its capacity to block Shh induction of motoneuron differentiation (3), did not prevent its effect on NCC migration (Fig. 4*C*; Table 1). Consistent with these observations, the mAb ASC1 was found to bind Shh to a site close to its N terminus and distinct from the site

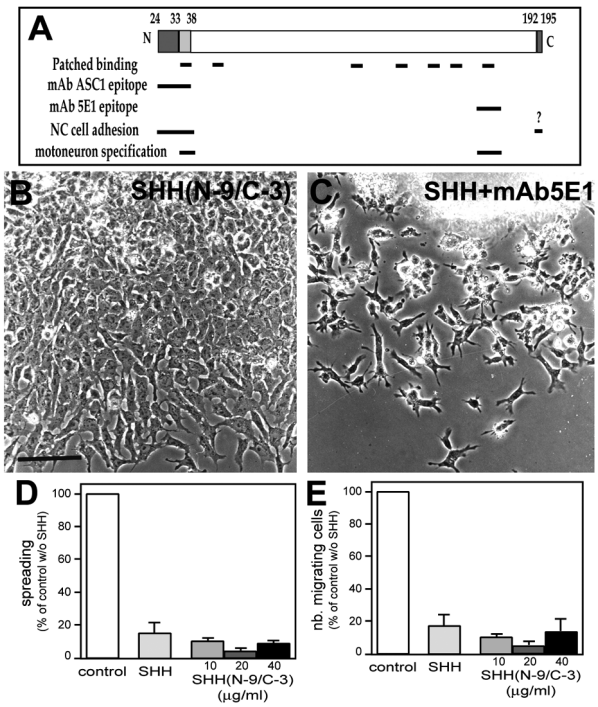

Fig. 4. Shh sequences involved in the control of NCC adhesion and migration. (*A*) Schematic representation of the Shh molecule showing the localization of the truncations in the Shh(N-9/C-3) mutant in dark gray and of the mutated sequence in Shh(PolyQ) in light gray. Putative regions recognized by the mAbs 5E1 and ASC1 as well as those engaged in Shh–Patched interactions mapped recently (38) are indicated. (*B* and *C*) NCC outgrowths on FN with Shh(N-9/C-3) at 20 μg/ml or on FN with Shh at 20 μg/ml in the presence of the mAb 5E1 at 150 μg/ml in the culture medium. (*D* and *E*) Effects of Shh at 20 μg/ml on cell spreading and migration in the presence of the Shh(N-9/C-3) used as a competitor on the substrate at increasing concentrations. The Shh(N-9/C-3) mutant is unable to compete with Shh for restoring NCC spreading and migration. (Bar = 50 μm.)

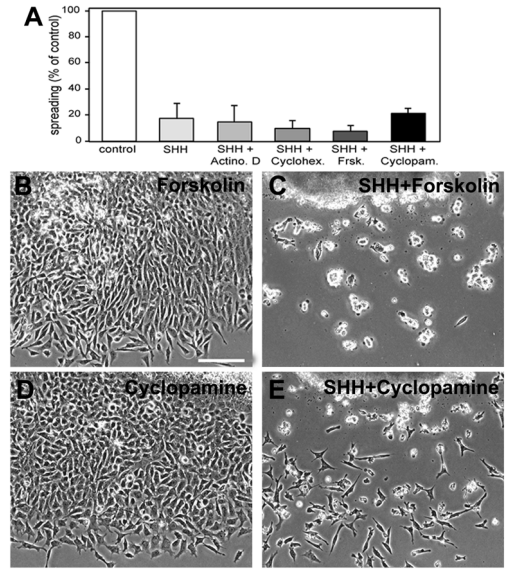

Fig. 5. The effect of Shh on NCC adhesion and migration is not mediated by the Smoothened-Gli signaling pathway. (*A*) Effects of actinomycin-D at 1 μg/ml, cycloheximide at 1 μg/ml, forskolin at 25 μM, and cyclopamine at 240 nM added to the culture medium on NCC spreading on FN in the presence of Shh at 20 μg/ml. Spreading was measured by using NCCs that have been collected from explants previously cultured on FN in the absence of Shh. (*B–E*) Effects of forskolin at 25 μM (*B* and *C*) and cyclopamine at 240 nM (*D* and *E*) on NCC migration from neural tube explants cultured on FN without (*B* and *D*) or with (*C* and *E*) Shh at 20 μg/ml. No reversion of the inhibition of NCC adhesion and migration can be observed when transcription or protein synthesis are blocked or when Smoothened and Gli activities are inhibited. (Bar = 50 μm.)

recognized by the mAb 5E1 (Fig. 4*A*). These data indicate that Shh sequences necessary for the regulation of cell adhesion map to its N terminus and differ at least partially from those implicated in motoneuron specification (Fig. 4*A*). To determine whether these sequences interact with the same region of the Patched receptor, we performed competition experiments between Shh and the Shh(N-9/C-3) mutant, taking advantage of the fact that this mutant binds Patched with the same apparent affinity as Shh. Results show that using the Shh(N-9/C-3) mutant as a competitor does not interfere with the effect of Shh on NCC adhesion and migration even at high concentrations (Fig. 4 *D* and *E*), suggesting that Shh sequences involved in control of cell adhesion and cell specification, respectively, interact with either separate domains within the Patched molecule or with distinct receptors.

Table 3. Functional properties of Shh and of mutated constructs and effects of mAbs on Shh

Treatment	Patched binding	Induction of motoneurons in neural plate explant	Inhibition of NCC migration
Shh	+++	+++	+++
Shh(polyQ)	–	–	–
Shh(N-9/C-3)	+++	+++	–
Shh + mAb ASC1	–	–	–
Shh + mAb 5E1	–	–	+++

Data on patched binding and induction of motoneurons in neural plate explants have been published elsewhere (17).

Beside the Patched protein, transduction of Shh signals involves the multipass membrane-spanning protein Smoothened as well as protein kinase A and the Gli family of transcription factors (26, 33). To determine whether Shh could employ this signaling pathway to repress integrin activity in NCCs, we first investigated the possible implication of Gli transcription factors. Forskolin, a well characterized activator of protein kinase A known to antagonize Shh signals notably in the neural tube (3), was not able to reverse the effect of Shh on NCC adhesion and migration (Fig. 5 *A–C*). Moreover, actinomycin D and cycloheximide, two inhibitors of gene transcription and protein synthesis, were also ineffective (Fig. 5*A*), suggesting that Gli-dependent transcriptional events are dispensable for the effect of Shh on cell adhesion. Next, we studied the effect of cyclopamine, a plant steroidal alkaloid that specifically inhibits the cellular response to Shh by acting on the Smoothened receptor (34). As shown in Fig. 5 *A*, *D*, and *E*, cyclopamine failed to interfere with the effect of Shh on NCC adhesion and migration. Collectively, these data strongly suggest that the effect of Shh on cell adhesion does not involve the canonical Patched-Smoothened-Gli signaling cascade.

Discussion

During vertebrate development the signaling molecule Shh plays a crucial role in the patterning of the nervous system as a morphogen, a mitogen and a trophic factor (26–28). Our present study reporting that Shh may regulate NCC adhesion and migration reveals a novel, unanticipated function of Shh in cellular interactions. Most importantly, this activity is discernable both in time and space from the other known functions in

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the neural tube and is not mediated by the Patched-Smoothened-Gli signaling pathway.

The action of Shh on NCC adhesion to extracellular matrices is sufficient to account for its dramatic effect on their dispersion as integrins are essential for NCC migration (13, 19, 25, 30). Preliminary data show that *in vivo* ectopic expression of Shh in the dorsal neural tube also leads to the accumulation of NCCs within the lumen of the neural tube (C.F.-T., A.J., and J.-L.D., unpublished data), a phenotype which can be ascribed to a block to cell migration and commonly observed in embryos where substrate adhesion of NCCs was prevented experimentally (12, 13). The question remains as to whether Shh can influence integrin function directly on NCCs themselves or indirectly by interfering with the activity of other signaling molecules such as BMPs. Several lines of evidence suggest that this effect might be essentially direct. First, we have not detected any significant changes in the expression patterns and apparent amounts of *Bmp4*, *Bmp7*,and *Noggin* in neural tubes confronted with Shh. Second, Shh was active within minutes in adhesion assays on isolated NCCs free of any neural tube influence. Third, its effect was fully reversible within less than 1 h. Therefore, if Shh directly acts on NCCs, what are the molecular mechanisms by which it regulates integrin function? One possibility is that Shh would inhibit integrin function via inside-out signals induced by surface receptors. Most intriguingly, our data suggest that, if it exists, this process does not rely on the signaling cascade elicited by the Patched and Smoothened receptors nor is it mediated by Gli transcription factors, unlike most biological activities of Shh described thus far. This observation would then indicate that Shh would bind to alternative receptors different from Patched. Another possibility is that Shh would affect integrin activity directly from the exterior of the cell without the implication of signaling events. Several arguments are in favor of such a mechanism. Shh had to be immobilized on the substrate to be effective, and the profile of the dose-response curve of cell adhesion was indicative of a competition effect. In addition, our preliminary data suggest that Shh is active on a broad range of cell types expressing β1 integrins but not on β1 integrin-deficient cells (A.J., C.F.-T., and J.-L.D., unpublished data). However, it is unlikely that Shh prevented adhesion by merely blocking access of integrins to extracellular matrix molecules, because its effect could be reverted either by adding specific Abs to Shh or by Mn^{2+} ions, and that it was equally active on extracellular matrix molecules differing by their repertoire of integrin receptors and on Abs to β1 integrins used as a substrate. Instead, we propose that Shh would interact with the β1-integrin chain either directly in its ligand-binding domain, thereby preventing its association with the extracellular matrix, or in another region of the molecule, thereby inducing a conformational shift toward an inactive state. Alternatively, Shh might interfere with the activity of integrin-associated transmembrane proteins known to modulate integrin function (35).

In a striking parallel with Shh, BMPs also contribute to the patterning of the neural tube (4) and act as potent inducers of NCC migration by modifying their adhesive properties (14, 15). Our present results reinforce the notion that the activities of Shh and BMPs on neuroepithelial cells are mutually exclusive, that they may share some common molecular targets and take place throughout the entire process of dorsoventral patterning of the nervous system. It is therefore conceivable that the role of Shh would be to overcome the scattering effect of BMPs in addition to antagonize their dorsalizing activity, thereby locally restricting the region where NCCs are formed and migrate while maintaining a high degree of cell–cell cohesion among ventral cells. On the other hand, it cannot be excluded that Shh could regulate adhesive properties of NCCs during later steps of their development, in particular during migration toward the ventral region of the embryo. It is now well established that NCCs systematically avoid the notochordal area because of repulsing cues contained in the perinotochordal matrix (36). Therefore, the effect of Shh on cell adhesion might also contribute to the guidance of NCCs during their migration. Interestingly, Shh has also been found to provoke the collapse of retinal ganglion cell axons (39). Consistent with this finding, activation of the EphA2 kinase implicated in the repulsive guidance of cell migration induces an inactive conformation of integrins and transiently inhibits cell spreading and migration in a very similar fashion to Shh (37).

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