Fascin, an Actin-Bundling Protein, Modulates Colonic Epithelial Cell Invasiveness and Differentiation *in Vitro*

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In epithelial tissue, cell-matrix and cell-cell adhesive interactions have important roles in the normal organization and stabilization of the cell layer. The malignant conversion of epithelial cells involves alterations in the expression and function of these adhesion systems that enable a switch to a migratory phenotype in tumor invasion and metastasis. Fascin is an actin-crosslinking protein that is found in the core actin bundles of cell-surface spikes and projections that are implicated in cell motility. We demonstrate that fascin is not detectable in normal colonic epithelium, but is dramatically up-regulated in colorectal adenocarcinoma. To test the hypothesis that fascin could participate in tumor invasive behavior, we developed a cell culture model to examine the effect of fascin expression on the adhesive interactions, invasiveness, and differentiation of colonic epithelial cells. We report marked effects on the organization of cell-surface protrusions, actin cytoskeleton, and focal adhesions in the absence of alterations in the protein levels of the major components of these structures. These effects correlate with alterations in cell movements on two-dimensional matrix, and increased invasiveness in three-dimensional matrix. The cells also show increased proliferation and decreased capacity for normal glandular differentiation in collagen gels. We propose that up-regulation of fascin, by promoting the formation of protrusive, actin-based, cell-motility structures, could be a significant component in the acquisition of invasive phe-

notype in colonic carcinoma. (*Am J Pathol* 2003, 162:69–80)

Epithelial cell differentiation is fundamentally influenced by cell-matrix and cell-cell interactions.^{1–3} In colonic epithelial cells, both the integrin and cadherin superfamilies of adhesion molecules are important contributors to the establishment of cell polarity and epithelial cell differentiation, and have been shown to play a role in the control of colorectal differentiation in tumor cells.^{4,5} This is partly achieved through the formation of intracellular protein assemblies that anchor cytoskeletal actin filaments at defined areas within the cell membrane. In epithelial cells, these zones correspond to integrin-dependent focal adhesions and cadherin-containing adherens junctions and desmosomes.⁶ These assemblies also function as important links in the integration of multiple cell signaling pathways.³ Cell-matrix and cell-cell adhesive interactions normally stabilize the epithelial cell layer and maintain the cells in a nonmigratory state. However, the malignant conversion of epithelial cells involves a phenotypic switch to a migratory state that enables tumor invasion beyond the basement membrane and metastasis. The process of cell migration is poorly understood in epithelial cells, but studies in many types of carcinoma cells have documented increased formation of cell protrusions at cell margins, release of cell-cell contacts, and group movement of sheets of cells. In the models of cell motility that have been developed from studies of fibroblasts, protrusion of a leading lamella and dynamic turnover of focal adhesions are key events that coordinate and integrate cell crawling on planar surfaces.⁷ These changes in motile behavior require complex rearrangements of the actin cytoskeleton that are governed by the functions of multiple actin-binding proteins.⁸ Of these proteins, fascin is an actin cross-linking protein that localizes to the core actin bundles of spikes and filopodia

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at the leading edge of migratory cells and that has been implicated in cell motility in several cell types.^{9,10} Although increased cell motility in cancer cells has been linked to decreased expression of several actin-associated proteins, including actinin and vinculin,¹¹ there are several reports that fascin expression is increased in certain cancers. Fascin expression is markedly increased in Epstein-Barr virus-transformed B lymphocytes and in the Reed-Sternberg cells of Hodgkin's lymphoma.^{12,13} Increased fascin expression has been reported in hormone receptor-negative breast carcinomas and in ovarian carcinomas.^{14,15} Given the known association of fascin with cell protrusions that are involved in cell motility, we wished to examine whether fascin could have a role in metastatic conversion of colonic epithelial cells. We report that fascin is strongly up-regulated in colorectal adenocarcinoma. To determine the functional consequences of fascin up-regulation in colonic epithelial cells, we have developed a set of cell lines that overexpress fascin and have analyzed its effects on the organization of adhesion complexes, proliferation, matrix attachment, invasive behavior, and differentiation in collagen gels. The results of our studies demonstrate that increased fascin expression in these cells correlates with increased cell invasiveness and proliferation and alterations in cell differentiation that involve a marked increase in protrusive matrix contacts. We discuss the implications of these results for further studies of epithelial cell motility and tumor invasiveness.

Materials and Methods

Cell Lines and Tissue Samples

A panel of gastric (AGS, HSC39, and Kato III), colonic (LIM1215, CaCo2, SW1222, SW948, SW480, SW620, HT29), and pancreatic (BxPC3, T3H4, Patu II, HPAF IV) carcinoma cell lines were cultured according to standard conditions. MKN7, and MKN45 were obtained from Riken Cell Bank, Ibaraki, Japan, by the kind permission of the originator, Dr. Teiichi Motoyama. HSC39 was kindly donated by the originator Dr. Kazuyoshi Yangihara (National Cancer Center Research Institute, Tokyo, Japan). LIM1215 was donated by Dr. Robert Whitehead (Vanderbilt University, Nashville, TN). The pancreatic cell lines were from Professor N. Lemoine, Cancer Research UK, Molecular Pathology Laboratory, Imperial College School of Medicine, Hammersmith Campus, London UK. All other cell lines were obtained from the European Tissue Culture Collection (Porton Down, UK). The SW1222 cell line that was selected for fascin transfection, was originally isolated from a primary adenocarcinoma of the colon (Dukes' stage C2, resected from a 44-year-old male).¹⁶ Paraffin-embedded sections were prepared from endoscopic biopsy samples of normal colonic mucosa obtained from patients with no malignancy and a series of colorectal adenocarcinomas and used for staining with fascin monoclonal antibody as described.¹² Ethical approval was obtained from the local research ethics committee of the United Bristol Health Care Trust. Rabbit

polyclonal antiserum to fascin¹⁷ was used for Western blotting and immunoprecipitation and a mouse monoclonal antibody obtained from DAKO was used for indirect immunofluorescence as previously described.^{18,19} Mouse monoclonal antibodies to E-cadherin, and α -, β -, and γ -catenin were purchased from Transduction Laboratories, Exeter, UK.²⁰ VIN 11.5 mouse monoclonal antibody to vinculin was purchased from Sigma Chemical Co.

Preparation of Stable Fascin Transfectant Cell Lines

SW1222 cells were transfected with the expression plasmid pcDNA3/human fascin-1, or empty pcDNA3 vector (Invitrogen) as a control.¹⁷ For transfection, a single cell suspension of the colonic epithelial carcinoma cell line SW1222, was prepared by trypsinization under calcium-free conditions. The cell suspension was passed through a 23-gauge needle three times and the formation of a single cell suspension confirmed by phase-contrast microscopy. Cells were plated at 10^6 cells per 90-mm dish 48 hours before transfection. Lipofection was performed using DOTAP (Boehringer Mannheim) according to the manufacturer's recommendations. Ten μ g of plasmid DNA was incubated with DOTAP in 20 mmol/L of HEPES buffer at room temperature for 10 to 15 minutes, then incubated with the cells for 6 hours in the absence of serum. Culture medium was then changed, and the cells maintained in standard medium containing 500 μ g/ml of geneticin (Sigma Chemical Co.). The optimal concentration of G418 for selection was taken to be the lowest concentration to cause 100% cell death by 2 weeks. Geneticin-resistant colonies were isolated by ring cloning and transferred to 24-well plates. The isolated colonies were screened for fascin expression by Western blotting. High-expressing clones were chosen for further assays.

Measurement of Cell Proliferation

Cell proliferation was assayed by 3 H-thymidine incorporation. Single cell suspensions were prepared and cell aliquoted into 96-well plates at 3×10^4 per well. After 12 hours of attachment, the cells were washed in serum-free medium and then incubated in medium containing 2% fetal calf serum for 24 hours, to bring the cells to G_0 . The cells were then transferred to medium containing 10% serum for 12 hours and then pulsed with 2 μ Ci 3 H-thymidine (AP Biotech) at a concentration of 20 μ Ci/ml for 8 hours. After washing in phosphate-buffered saline (PBS) the cells were lysed in 200 μ l of 1 mol/L KOH. The radioactivity of each sample was determined using an LKB 1216 liquid scintillator counter. Assays were performed in triplicate. Cell proliferation was also assessed by staining cells cultured in three-dimensional collagen gels for Ki67 nuclear antigen, using MIB I antibody. The cell-labeling index was calculated by counting the number of positively staining nuclei in a total of 1000 cells per sample.

Immunofluorescent Staining of Adherent Cells

Fascin transfectant and vector-control SW1222 clonal cell lines, grown at low density on laminin-precoated glass coverslips, were fixed in ice-cold methanol for staining with fascin and β -catenin monoclonal antibodies (Affiniti Labs) as previously described,^{18,19} followed by detection with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (ICN Biochemicals). For F-actin or vinculin stainings, cells were fixed in 10% formaldehyde followed by permeabilization in buffer containing 50 mmol/L 4-morpholineethanesulfonic acid, pH 6.1, 5 mmol/L $MgCl_2$, 3 mmol/L [ethylenebis(oxyethylenetriole)]tetraacetic acid, 100 mmol/L KCl, and 0.2% Triton X-100. Permeabilized cells were then stained with tetramethyl-rhodamine isothiocyanate-phalloidin, to visualize F-actin or incubated with appropriate fluorescein isothiocyanate-conjugated secondary antibody. Staining patterns were examined by epifluorescence using a Zeiss Axiophot microscope and photographs were taken on Kodak Tmax 100 film.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

Cell extracts were prepared according to the method of Laemmli.²¹ Extracts equalized by protein content were resolved on 8 or 10% polyacrylamide gels under reducing conditions. The separated proteins were transferred to 0.22- μ m pore nitrocellulose (Bio-Rad). Matched protein loading was confirmed using Ponceau S staining. Membranes were blocked and incubated in primary antibody at 4°C overnight. After washing in Tris-buffered saline containing 0.5% Tween-20, the membrane was incubated with species-specific anti-immunoglobulin coupled to horseradish peroxidase (DAKO) at room temperature for 1 hour. Immunoreactive bands were detected by ECL chemiluminescent reagent (Amersham) and exposure to Kodak Biomax X-ray film.

Immunoprecipitation

Extracts for immunoprecipitation were prepared as described in Hinck and colleagues.²² Briefly, cells were solubilized in Triton X-100 lysis buffer (50 mmol/L NaCl, 10 mmol/L Pipes, pH 6.8, 3 mmol/L $MgCl_2$, 0.5% Triton X-100, 300 mmol/L sucrose) with protease inhibitors for 20 minutes on ice. Protein G-Sepharose beads (Pharmacia), were incubated with cell extract in the presence of fascin antiserum or nonimmune control serum overnight at 4°C. The samples were then washed repeatedly and beads pelleted by centrifugation. The precipitated proteins were eluted from the beads by boiling in Laemmli sample buffer for 5 minutes. Precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting as described above.

Quantitation of Cell-Matrix Attachment

Cell-matrix attachment was quantitated as described in Hudson and colleagues.²³ Briefly, bacteriological microtiter plates (Flow Laboratories, Oxfordshire, UK), were coated with collagen type IV or laminin (Sigma Chemical Co.) at 5 μ g/ml or bovine serum albumin at 5 mg/ml. Previous experiments have determined these concentrations of laminin or collagen IV to be appropriate for maximal adhesion of SW1222 cells.⁵ Single cell suspensions of SW1222 clones were prepared and 5×10^4 cells added per well and incubated for 3 hours at 37°C to allow cell attachment. Nonadherent cells were removed by gentle washing in PBS and the number of adherent cells calculated using a substrate-induced color change assay that involved the addition of 3.75 mmol/L of *p*-nitrophenyl-*N*-acetyl- β -glucosamine in citrate buffer. Cellular adherence was determined by assay of hexosaminidase activity by measuring absorbency at 405 nm using a microtiter plate reader (Titertek Multiskan Plus). The optical density was calibrated to cell number by measuring color development on known numbers of suspended cells in the range 3×10^2 to 5×10^4 cells. All assays were performed in triplicate.

Transfilter Invasion Assay

Cell culture inserts (Falcon, Becton Dickinson) containing Transwell filters with 8- μ m pores were precoated on both surfaces with collagen type I or collagen type IV (both at 5 μ g/ml), by incubating overnight at 4°C. and then incubated in 5 mg/ml of bovine serum albumin for 1 hour to block nonspecific binding. Two ml of serum-free media containing a total of 1×10^6 cells were placed in the top compartment, and incubated at 37°C for 6 hours. Cells were fixed in 100% methanol for 10 minutes and the top surface of the filter wiped to remove all adherent cells. Cells that had migrated to the lower surface of the filter were stained and visualized with hematoxylin. Cell migration was quantified by counting the number of cells in nine fields of view per filter, using $\times 25$ magnification. Assays were performed in triplicate.

Time-Lapse Videomicroscopy

Single cell suspensions were prepared and cells were plated overnight at 3×10^4 cells/flask in Nunc Slide Flasks in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Videomicroscopy was performed in a 37°C environmental chamber using a Zeiss Axiovert 100 microscope, fitted with a Sony SS-M370CE charge-coupled device camera linked to a videorecorder and driven by an EUS BAC900 animation controller as described.¹⁹ Migration was recorded at four frames per minute throughout 6- to 8-hour periods and the move-

ment patterns of individual cells or colonies were assessed by direct measurement from traces.

Analysis of Cell Differentiation in Three-Dimensional Collagen Gel

Collagen type I gels (Vitrogen 100; Celtrix, Santa Clara, CA) were prepared according to the manufacturer's recommendations. A single cell suspension of SW1222 cells was prepared by trypsinization and added to neutralized collagen/Dulbecco's modified Eagle's medium solution (9:1 ratio by volume) to give a final density of 2×10^6 cells/ml of collagen. The mixture was aliquoted into pre-warmed 24-well culture plates and set in a CO₂-free incubator at 37°C. Two ml of culture medium was added per well and was changed three times per week. The gels were incubated for 3 to 4 weeks to allow assessment of the development of three-dimensional colony morphology and cell proliferation as previously described.²⁴ The gels were fixed in formalin, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin and with Alcian Blue/periodic acid-Schiff staining for mucins. Other sections were immunostained for E-cadherin and catenins.²⁰

Electron Microscopy

SW1222 cells were allowed to grow on plastic for 2 weeks to form large mature colonies, and then detached with nonenzymatic dissociation media (Sigma C-5914). This allowed preservation of cell-cell interactions, while dissociating the cells from underlying plastic and accumulated matrix. Colonies were fixed in 2% glutaraldehyde for 2 hours at 4°C, transferred to PBS, and postfixed in 1% phosphate-buffered osmium tetroxide for 1 hour at 4°C with rotation. Fixed colonies were rinsed in distilled water and then dehydrated in graded alcohols (30%, 50%, 70%, 90%, and 100% twice), for 30 minutes at each step. Colonies were then soaked with TAAB resin (TAAB, Berkshire, UK) and allowed to stand for 1 hour with rotation. The resin was changed twice and the cells then embedded in fresh resin in beam capsules, and polymerized at 60°C overnight. The polymerized blocks were trimmed and 1-mm sections were cut with a glass knife and stained in toluidine blue. Suitable blocks for electron microscopy were chosen and 90-nm ultrathin sections were cut using a diamond knife, spread with chloroform, and collected on nickel grids. The sections were counterstained with uranyl acetate and examined under a Philips CM10 transmission electron microscope.

Statistical Analysis

Data are expressed as mean $\pm$ SE. Statistical analysis was performed by Student's *t*-test and analysis of variance. A *P* value of <0.05 was considered statistically significant.

Figure 1. Fascin immunoreactivity in normal colonic mucosa (**A**) and colonic adenocarcinoma (**B**). Overexpression of fascin is detected in colonic carcinoma cells.

Results

Up-Regulation of Fascin in Colonic Adenocarcinomas

We examined the distribution of fascin in normal colonic mucosa by antibody staining. The colonic epithelial cell layer appeared negative for fascin. Positive staining was apparent in the underlying lamina propria, within endothelial cells, lymphocytes, and fibroblasts (Figure 1A). In sharp contrast, colorectal adenocarcinoma cells displayed strong, uniform fascin staining throughout cell bodies (Figure 1B, shown for 1 representative tumor sample of 10). Staining was also present in the surrounding stroma, including endothelial cells and inflammatory cells (Figure 1B). These results clearly demonstrate up-regulation of fascin as a phenotypic alteration in colonic carcinomas.

Generation of Fascin-Overexpressing Colonic Epithelial Cells

To determine the consequences of fascin up-regulation on colonic epithelial cell interactions and behavior, we developed a cell culture model of matched cell lines positive or negative for fascin. We first screened a panel of gastric, colonic, and pancreatic cell lines for fascin expression by Western blotting. SW1222, Kato III, and HSC39 were negative or had barely detectable levels of

Figure 2. A: Western blot for fascin expression in a panel of gastric, colonic, and pancreatic cell lines. Kato III, HSC39, and SW1222 had low or undetectable fascin. **B:** Western blot of fascin expression in SW1222 transfectant clonal lines. Clones C9 and C11 were chosen for further examination.

fascin expression. All other cell lines tested contained varied levels of fascin protein and there was no overall correlation between the level of fascin and differentiation status. Similar results have been documented in breast cancer cell lines, which, although tumor-derived, have been in culture for many years (Figure 2A).²⁵ To determine the effects of *de novo* fascin expression in a uniform genetic background, SW1222 was chosen for transfection studies because it is a well-differentiated colonic epithelial cell line that has been well-characterized. The cells form organized aggregates in culture in which cells make tight cell-cell contacts and show expression and function of the E-cadherin-catenin complex equivalent to normal colonic epithelial cells.^{5,26} From the transfections, two clonal derivatives that expressed stable, highly elevated levels of fascin were chosen for further investigation: these lines were designated SW12C9 and SW12C11 (Figure 2B). A number of vector control clones were also isolated and one of these, VC1, was used as a control in all subsequent experiments (Figure 2B).

Effects of Fascin Expression on Proliferation of Colonic Epithelial Cells

We noted an apparent increase in cell proliferation during day to day culture of the fascin transfectant cell lines, compared to VC1 and other control clones. To explore this further, DNA synthesis was measured by tritiated thymidine uptake in the C9 and C11 lines in comparison with clone VC1. A statistically significant increase in cell proliferation was apparent in both transfectant cell lines compared to vector control showing a 10-fold increase in thymidine uptake in C9 and sixfold in C11 compared to

Figure 3. Analysis of Ki67-expressing cells in three-dimensional colonies. Sections were stained with MIB I antibody to Ki67: VC1 (A), C9 (B), and C11 (C). Scale bar, 100 μ m.

VC1. This increase in the proliferation of cycling cells was further confirmed by staining colonies grown in three-dimensional collagen gels for expression of the nuclear antigen Ki67 (Figure 3). One thousand cells were counted in each sample and the proportion of positively staining nuclei expressed as a percentage (the labeling index). The VC1 line had a labeling index of 23% com-

pared to 52% and 74% in the C9 and C11 cell lines, respectively ($P < 0.001$).

Fascin Up-Regulation Affects β_1 Integrin Subcellular Localization But Not E-Cadherin/Catenin Complex

The morphology of the transfectant cell lines in culture was first assessed by phase-contrast light microscopy. The morphology of the fascin-expressing clones differed from that of parental or vector control VC1 cells in several respects. Wild-type and VC1 cells grow in tightly compacted colonies that became several cell layers thick in the center (Figure 4A). Clones SW12C9 and SW12C11 formed more angular colonies in which cells at the edges in contact with the substratum appeared more spread and exhibited many spiky peripheral projections (Figure 4, B and C, arrows). Cells at the edges of the colonies also appeared more well spread than VC1 cells. We therefore examined the assembly and localization of cell contacts in more detail by staining for major structural components. With regard to the assembly of cadherin-based cell-cell contacts, we examined the distribution of β -catenin in low-density colonies of the transfectant cell lines. In VC1 colonies and in the two transfectant cell lines C9 and C11 the cells displayed clear concentrations of β -catenin at cell-cell junctions within the colonies (Figure 5; A to D) as previously seen in the SW1222 parental cell line.²⁴ Although C9 and C11 colonies were more angular than VC1 colonies (Figure 5, C and D), β -catenin staining remained concentrated at cell-cell junctions. The distribution of E-cadherin was equivalent to that of β -catenin in all of the cell lines (data not shown). Because β -catenin has been reported as a second binding partner for fascin,²⁷ we examined the effects of fascin transfection on the expression of major proteins of the E-cadherin-catenin complex and on the possible association of fascin with this complex. By Western blot, fascin transfectant cells showed no alteration in E-cadherin, or α -, β -, or γ -catenin level and no change in the amount of E-cadherin that co-precipitated with β -catenin was apparent between the cell lines (data not shown). We then examined the distribution of β_1 integrin subunit, as a common component of the major integrins of colonic epithelial cells.⁵ In parental and VC1 cells, β_1 integrins appeared in part diffusely distributed and in part concentrated at cell-cell junctions, as is typical in epithelial cells (Figure 6, A and B). In C9 and C11 cells, concentrations of β_1 integrin were also localized to the outer margins of cells at the edges of colonies, including the peripheral projections (Figure 6, C and D). By immunoblot, there was no difference in β_1 integrin expression (data not shown).

Up-Regulation of Fascin Correlates with Reorganizations of the Actin Cytoskeleton and Focal Adhesions

The increase in cellular protrusions and altered localization of β_1 integrin in the fascin-overexpressors lines sug-

Figure 4. Phase-contrast images of VC1 (A), C9 (B), and C11 (C) cell lines. With reference to VC1, cells in B and C show typical changes in colony morphology including increased spreading of marginal cells (arrows in B) and cellular projections (arrows in C). Scale bar, 100 μ m.

gested that fascin possibly affected cell-matrix contacts. We therefore examined these structures in more detail by staining for F-actin and for vinculin, a prominent component of focal adhesions. Phalloidin staining of VC1 cells showed concentrations of F-actin at cell-cell junctions and in the small ruffled lamellipodia of some marginal cells within the colonies (Figure 7A). In contrast, C9 or C11 cells showed concentrations of F-actin at the tips of finger-like protrusions and striking bands of F-actin at the free edges of substratum-adherent cells at the margins of colonies (Figure 7, B and C). We examined the localization of vinculin, as a structural marker for focal adhesions. Typically, epithelial cells do not assemble prominent fo-

Figure 5. Analysis of β -catenin cellular localization in laminin-adherent fascin transfectants. VC1 (**A**, **B**), C9 (**C**), C11 (**D**). Scale bar, 10 μ m.

cal contacts and indeed the localization of vinculin in VC1 was mainly diffuse, with a few spot-like concentrations in cells at the edges of colonies (Figure 8A). Unexpectedly, large numbers of focal contacts were assembled by C9 and C11 cells (Figure 8B). These contacts were particularly prominent in the well-spread cells at the margins of colonies, but were also present in cells within the central regions of colonies. Detailed examination of the distribution of focal contacts at cell margins indicated that contacts were formed where the peripheral projections contacted the substratum. In isolated, morphologically polarized C11 cells, focal contacts were prominent both at the leading edge and within the retracted, tail region (Figure 8D). Because of the increased prominence of these focal adhesions, we compared vinculin protein expression between the cell lines. No alteration in the level of vinculin protein was detectable (data not shown). In C9 and C11 cells fascin was diffuse within cell bodies and was also present in cell-surface projections (Figure 8, E and F). Together, these observations suggest that fascin up-regulation alters focal adhesion assembly and induces reorganization of the actin cytoskeleton, without major effects at the cell-cell contacts.

Up-Regulation of Fascin in SW1222 Increases Cell Motility and Invasion

The altered distribution of β 1 integrin in the fascin over-expressor lines suggested that the interaction with extracellular matrix might be altered. To test this directly, we quantified the initial attachment of the SW12VC1 clonal lines to collagen IV or laminin-1, which are the major components of the basement membrane of the colonic epithelium. The fascin-overexpressing cells showed slightly enhanced attachment to collagen IV, but this difference was not statistically significant (Figure 9A). There was no alteration in cell attachment to laminin compared to VC1 (Figure 9B). Fascin is associated with cell motility structures in several cell types^{9,10,19,27,28} and its overexpression in SW1222 also correlated with the formation of cell protrusions. Time-lapse videomicroscopy showed that both C9 and C11 cells typically exhibited dynamic membrane activity with repeated and rapid extension and retraction of large, blunt, finger-like projections from the edges of the cells (data not shown). The appearance of these membranous projections did not

Figure 6. Analysis of β_1 -integrin cellular localization in laminin-adherent fascin transfectants. VC1 (**A**), C9 (**B**), C11 (**C**, **D**). **Small arrows** indicate concentration of β_1 integrin at cell-cell borders. Scale bar, 10 μ m.

Figure 7. Analysis of cytoskeletal organization in laminin-adherent fascin transfectants. Small colonies of VC1 (**A**) and C11 (**B**) were stained with tetramethyl-rhodamine isothiocyanate-phalloidin. **Small arrows** in **B** indicate presence of F-actin at the free edges of adherent cells at the margins of colonies. Scale bar, 10 μ m.

have a simple correlation with progressive cell migration over the surface, very likely because the cell-cell adhesions prevented extensive migration.²⁵ Cells in colonies underwent small circling movements, whereas isolated cells that exhibited a clear morphological polarity locomoted extensively across the surface. These alterations in cell behavior were quantified from traces of 50 cells in each population. In VC1 only 8% of the cells showed some degree of membrane movement at cell margins. In contrast, C9 and C11, which exhibited spiky elongated cellular projections, showed a significant increased dynamic membrane activity, with extension and retraction of finger-like projections in more than 80% of cells ($P < 0.001$). The acquisition of invasive behavior by epithelial cells involves penetration of the basement membrane and subsequent migration within the underlying collagen-rich stroma. We therefore examined the effects of fascin overexpression in a cell invasion, transwell filter assay that used single cells. Clones C9 and C11 showed a twofold to fivefold increase in migration through filters

Figure 8. Analysis of vinculin and fascin cellular localization in laminin adherent fascin transfectants. Small colonies of VC1 (**A**), C9 (**B**, **E**), and C11 (**C**, **D**, **F**) were stained for vinculin (**A–D**) and fascin (**E**, **F**). **Arrows** in **B**, **C**, **D**, and **F** indicate the increased assembly of focal contacts by single cells or cells in colonies. **Large arrow** in **F** indicates apical cluster of fascin projections. Scale bar, 10 μ m.

uniformly coated with collagen types I or IV, compared to VC1 cells (Figure 9C). This difference was statistically significant ($P < 0.05$). Thus, by several parameters, fascin overexpression correlated with increased motile behavior.

Up-Regulated Fascin Expression Perturbs Functional Differentiation in Three-Dimensional Matrices

These alterations in behavior were further examined by culturing the cell lines embedded in collagen gels for 3

weeks. Under these conditions, parental SW1222 cells grow as cell aggregates that undergo glandular differentiation as manifested by polarization of nuclei toward the basal surface of cells and the organization of cells around a central lumen (Figure 10A).²⁶ In comparison with the VC1 line, which grew indistinguishably from parental SW1222, colonies of C9 and C11 cells tended to be larger and show less organized glandular architecture, with development of multiple tubular lumina within each colony and increased secretion of a mucinaceous substance within these lumina (Figure 10, B and C). To examine differentiation status in more detail, sections of

Figure 9. Cell-matrix adhesion and migration on different extracellular matrix proteins of fascin transfectants (VC1, C9, and C11 cell lines). Quantitation of attachment to collagen IV (**A**) and laminin (**B**) under serum-free conditions. Values are means $\pm$ SE. No significant difference in adhesion was observed. **C:** Cell migration on different extracellular matrix proteins of fascin transfectants (VC1, C9, and C11 cell lines). Quantitation of migration across collagen IV (**C**)-coated filters. Values are means $\pm$ SE.

the cultures were stained with Alcian Blue/periodic acid-Schiff to visualize the mucins, major differentiation products of colonic epithelial cells. Acidic mucins are identified by the interaction of acidic side chains with cationic basic dyes such as Alcian Blue. Neutral mucins lack these acidic side groups and hence do not react with Alcian Blue. These mucins are identified by the periodic acid-Schiff stain, which produces a magenta color product. A predominant increase in the neutral (magenta) and to a lesser extent of acidic (blue) mucins was apparent in C9 and C11 (Figure 10, E and F) cultures relative to VC1 (Figure 10D). The ultrastructure of cell-cell junctions within colonies was examined by transmission electron microscopy. Cell junctional structures, including adherens junctions and desmosomes, were apparent in all of the cell lines (Figure 11, A and B). Aggregates of cells around mucin-containing lumina mimicked glandular structures in which the apical (ie, luminal) borders of the cells were covered with microvilli (Figure 11A). In C11 cells, parallel arrays of actin microfilaments were particularly evident within the cell surface microvilli (Figure 11B). These results, in combination with our immunostaining and biochemical data, demonstrate that fascin overexpression does not lead to major changes in the assembly of cell-cell contacts.

Figure 10. Organization of cell colonies in three-dimensional collagen gels. VC1 (**A, D**), C9 (**B, E**), and C11 (**C, F**) were grown in collagen gels for 3 weeks, fixed, sectioned, and stained with H&E (**A–C**) or Alcian Blue/periodic acid-Schiff (**D–F**) stain to visualize mucins. C9 and C11 showed increased production of both acidic (blue stain, **arrows** in **E**) and neutral mucin (magenta stain, **arrows** in **F**). Scale bar, 100 μ m.

Discussion

We report here that the actin cross-linking protein fascin is up-regulated in human colorectal adenocarcinoma compared to the normal colonic epithelium. To demonstrate whether fascin could have a functional effect on colonic epithelial cell behavior that might be involved in tumor invasiveness, we developed fascin overexpressor cell lines using SW1222, a well-differentiated colonic carcinoma derived cell, which has low endogenous fascin and normal cell-adhesion properties. This model provided us with a clearer basis for analyzing the functional consequences of fascin overexpression than compari-

Figure 11. Analysis of colony organization by transmission electron microscopy. Ultra-thin sections of C11 cells counterstained with uranyl acetate. **A:** Section at cell-cell junction. **B:** Section across glandular lumen (L). AJ, adherens junction; MV, microvilli; MF, actin microfilament. Original magnifications: $\times 35,625$ (**A**); $\times 14,375$ (**B**).

sons between existing tumor lines from heterogeneous sources. The major results of our experiments are that fascin overexpression correlates with increased formation of dynamic cell protrusions, alterations in the structural assembly of focal adhesions, increased proliferation, and increased invasiveness in several experimental contexts. These results are novel and indicate a possible role for fascin in the development of colonic carcinoma.

A striking feature of the fascin overexpressor cells was the increased formation of actin- and fascin-containing surface protrusions. Detailed study by time-lapse videomicroscopy showed that these structures were dynamic and rapidly extended and retracted over the substratum. The presence of β_1 integrin and associated focal adhesions in these structures suggested that the formation of the protrusions might be mediated by alterations in integrin-attachment properties. However, we found no significant alteration in cell attachment to laminin or collagen IV. The extent of integrin-cytoskeletal linkage is known to regulate migratory behavior.²⁹ In other systems, development of tractional force across focal adhesions has been correlated with the extension of protrusions.³⁰ Formation of protrusive structures that are regulated separately from cell body adhesion have been reported in several tumor cell types^{31,32} and it seems likely that in SW1222 cells, fascin contributes to the formation of this general type of cell-motility structure.

As in the colorectal adenocarcinoma samples, diffuse fascin staining was apparent in the majority of cancer cells. Matrix adhesion is known to regulate the phosphorylation status of fascin and its association with F-actin¹¹ and it is likely that, under the assay conditions used, the cells contain both forms of fascin. A second obvious effect of fascin overexpression was on colony organization, both on a laminin substratum and within three-dimensional collagen gels. The expression of epithelial polarity and glandular organization by SW1222 cells is a specific response to collagen gels that depends on the adhesive activities of E-cadherin and the $\alpha 2 \beta 1$ integrin.⁵ Fascin overexpressors underwent polarization and differentiation, as demonstrated by staining and transmission electron microscopy studies for cell-cell adhesions, however overall colony organization and other aspects of the differentiated phenotype appeared perturbed. This may be a consequence of the greatly increased percentage of cycling cells within each colony. Although up-regulation of fascin has been reported in several forms of carcinoma and lymphoma, to our knowledge this is the first report of an effect of fascin on epithelial cell proliferation.^{9,13-15} Studies to establish the mechanism involved are underway. A second particularly interesting aspect was the elevated level of neutral mucin within the glandular lumina. Mucins are secreted from gastrointestinal epithelium and their structure and composition vary according to the state of cellular differentiation.³³ It has been shown that acid mucin is a major component of normal gastrointestinal epithelial cells whereas neutral mucins are found in adenomas and in early colonic malignancies with less differentiation.^{34,35} The significance of this unexpected finding is as yet unclear, but raises a number of interesting possibilities. Fascin overexpression could be

responsible for an increase in cell proliferation resulting in a less differentiated phenotype. Further investigation of MUC gene expression in these transfectants is now underway.

Because β -catenin has been reported as a second binding partner for fascin, we gave particular attention to the effects of fascin on cadherin-based cell-cell contacts. As demonstrated by transmission electron microscopy and immunofluorescent staining studies, fascin overexpressor cells were not affected in their ability to assemble appropriately located adherens junctions or to localize E-cadherin and β -catenin to cell-cell margins. Furthermore, at the biochemical level, the amount of E-cadherin associated with β -catenin was not altered. Thus, the presence of fascin did not alter the ability of SW1222 cells to form the protein complexes necessary for the adhesive function of E-cadherin.³ An association of fascin with β -catenin was detectable in the overexpressor lines but, as described above, did not correlate with obvious alterations in the assembly of E-cadherin complexes. We conclude that the effects of fascin on cell organization and behavior are not mediated by effects on the organization of cell-cell adhesion complexes.

Although fascin-overexpression correlated with the formation of dynamic cell protrusions and isolated cells were more migratory on two-dimensional matrix and in an invasion assay, the presence of these finger-like protrusions did not show a clear correlation with increased locomotion for cell colonies on planar substrata. An inverse correlation between focal adhesion density and cell locomotion has been documented in fibroblasts.³⁶ The initiation of cell locomotion in epithelial cells also requires regulated alterations in focal adhesion assembly and loss of cell-cell adhesions. The lack of sustained migration by fascin overexpressors in colonies may thus relate both to the presence of focal adhesions and the maintenance of functional cell-cell contacts. It will be of future interest to determine the molecular mechanism by which fascin regulates invasiveness. The role of phosphatidylinositol 3-kinase is of particular interest, because of its involvement in the IGF-induced colony dispersion of breast cancer cells.²⁵ There is evidence that cell adhesion and migration in three-dimensional matrix may rely on alternate forms of matrix contacts to crawling locomotion on rigid, planar surfaces where focal adhesions predominate.^{10,37} Tumor cells in three-dimensional matrix show a particular propensity to form protrusive structures termed invadopodia and pseudopodia that are implicated in cell motility.¹⁰ We propose that fascin, by participating in the formation of cell protrusions, may promote invasion and metastasis of colonic cancer cells and be associated with a more aggressive phenotype and clinical behavior.

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