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Phosphoinositide-3 Kinase–Rac1–c-Jun NH_2-terminal Kinase Signaling Mediates Collagen I–induced Cell Scattering and Up-Regulation of N-Cadherin Expression in Mouse Mammary Epithelial Cells[D]

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During epithelial-to-mesenchymal transitions (EMTs), cells must change their interactions with one another and with their extracellular matrix in a synchronized manner. To characterize signaling pathways cells use to coordinate these changes, we used NMuMG mammary epithelial cells. We showed that these cells become fibroblastic and scattered, with increased N-cadherin expression when cultured on collagen I. Rac1 and c-Jun NH_2-terminal kinase (JNK) were activated when cells were plated on collagen I, and dominant inhibitory Rac1 (RacN17) or inhibition of JNK signaling prevented collagen I–induced morphological changes and N-cadherin up-regulation. Furthermore, inhibiting phosphoinositide-3 kinase (PI3K) activity prevented Rac1 and JNK activation as well as collagen I–induced N-cadherin up-regulation. These data implicate PI3K–Rac1–JNK signaling in collagen I–induced changes in NMuMG cells. To establish a role for N-cadherin in collagen I–induced cell scattering, we generated N-cadherin overexpressing and knockdown NMuMG cells and showed that knocking down N-cadherin expression prevented collagen I–induced morphological changes. Motility assays showed that cells overexpressing N-cadherin were significantly more motile than mock-transfected cells and that N-cadherin-mediated motility was collagen I dependent. In addition, we showed that cord formation and branching in three-dimensional culture (EMT-dependent events) required N-cadherin expression and PI3K–Rac1–JNK signaling.

INTRODUCTION

Cadherins and integrins mediate cell–cell and cell–extracellular matrix (ECM) interactions, respectively, and play important roles during cell proliferation, differentiation, survival, migration, and gene expression (Hynes, 2002; Wheelock and Johnson, 2003). Coordination of the signals cells receive from cadherins and integrins is essential for the cellular movements that contribute to both normal development and to cancer cell metastasis (Brunton *et al*., 2004; Derycke and Bracke, 2004). Previous studies have suggested cross-talk between these two adhesion systems. For example, during keratinocyte terminal differentiation, E-cadherin was shown to play a role in the down-regulation of $\alpha 6\beta 1$ integrin (Hodivala and Watt, 1994), and the introduction of E-cadherin into fibroblasts caused down-regulation of $\alpha 3\beta 1$ integrin and reduced adhesion to ECM (Finnemann *et al*., 1995). In addition, $\beta 1$ and $\beta 3$ integrins regulate the surface distribution and activity of N-cadherin in migrating neural crest cells (Monier-Gavelle and Duband, 1997). Kawano *et al*. (2001) showed that plating squamous epithelial cells on type V laminin disrupted E-cadherin–mediated intercellular adhesion and implicated integrin $\alpha 3\beta 1$ in this activity. Likewise, integrin $\beta 1$ was shown to regulate the polarity and motility of epithelial cells by down-regulating cadherin function and activating Rac1 and RhoA (Gimond *et al*., 1999). In Madin-Darby canine kidney (MDCK) cells, activating Rac by expressing Tiam1, a Rac guanine nucleotide exchange factor, or by expressing constitutively active Rac had opposing outcomes, depending on the substrate the cells were plated on. When plated on fibronectin or laminin, the cells showed decreased motility and increased cell–cell adhesion, whereas cells plated on collagen I had increased motility rates (Sander *et al*., 1998). Furthermore, the nonreceptor tyrosine kinase Fer and the serine/threonine kinase integrin-linked kinase have been shown to regulate both cadherin and integrin function (Wu *et al*., 1998; Arregui *et al*., 2000). Thus, a number of studies have implicated cadherins in regulating integrin function and integrins in regulating cadherin function. In addition, select signaling molecules have the ability to impact both integrin and cadherin activity, making it clear that cells have mechanisms to promote cross-talk between these two adhesion systems. However, the details of the signaling pathways that promote this cross-talk are not yet understood and are likely different among diverse systems.

When epithelial cells change their relative position within a tissue, they convert to motile fibroblastic cells. This phenomenon is referred to as an epithelial-to-mesenchyme transition (EMT) (Affolter *et al*., 2003). EMT is often accompanied

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by loss of E-cadherin and increased expression of other cadherins, such as N-cadherin, K-cadherin, or R-cadherin, depending on the tissue type (Thiery, 2003). When cancer cells invade adjacent tissues, they use a mechanism akin to EMT, and loss of E-cadherin expression in epithelial carcinomas is thought to be the primary reason for disruption of tight epithelial cell–cell contacts and release of invasive tumor cells from the primary tumor (Thiery, 2002; Wheelock and Johnson, 2003). We have shown that, in addition to the loss of the invasion-suppressor E-cadherin, another adhesion molecule, N-cadherin, becomes up-regulated in invasive tumor cells (Islam *et al*., 1996). N-cadherin promotes cell motility, which is critical to tumor invasion and metastasis (Nieman *et al*., 1999; Hazan *et al*., 2000) and E- to N-cadherin switching is one step in the formation of invasive tumorigenic cells (Cavallaro *et al*., 2002; Christofori, 2003). Furthermore, recent studies from our laboratory showed that cadherin switching is necessary for the increased cell motility that accompanies transforming growth factor β (TGFβ)–induced EMT in mammary epithelial cells (Maeda *et al*., 2005).

Maintenance of epithelial tissues requires interactions with the stroma. In cancer, changes in the stroma drive invasion and metastasis, the hallmarks of malignancy (De Wever and Mareel, 2003). In addition, during oncogenic transformation, integrin-mediated responses to ECM can result in loss of cell polarity, dedifferentiation of epithelial cells, and a switch to a more motile invasive phenotype (Keely *et al*., 1997). Some cell lines, such as NBT-II, respond to inducers of EMT much more rapidly when they are cultured on type I collagen rather than on other substrata (Tucker *et al*., 1990; Savagner, 2001). Thus, it is clear that when cells undergo phenotypic changes such as EMT, they must coordinate changes in cell–cell interactions, such as cadherin switching, with changes in cell–substrate interactions. Our hypothesis for the current study was that integrin-mediated cell motility must be coordinated with N-cadherin up-regulation through EMT-related signaling pathways.

MATERIALS AND METHODS

Reagents, Antibodies, and Cultured Cells

All reagents were from Sigma-Aldrich (St. Louis, MO) or Fisher Chemicals (Fair Lawn, NJ) unless otherwise indicated. Mouse monoclonal antibodies (mAbs) against the cytoplasmic domains of E-cadherin (4A2) and N-cadherin (13A9) have been described previously (Johnson *et al*., 1993). Rat mAb against the extracellular domain of mouse E-cadherin (ECCD2) was from Zymed Laboratories (South San Francisco, CA). Anti-Integrin β1, anti-paxillin, anti-Rac, anti-focal adhesion kinase (FAK), anti-Cdc42, anti-phosphotyrosine (PY20), anti-smad2/3, and anti-Akt mouse mAbs were from BD Biosciences PharMingen, Bedford, MA. Anti-FAK phospho-specific (Tyr577) and anti-c-Jun NH_2-terminal kinase (JNK) phospho-specific (Thr183/Tyr185) rabbit polyclonal antibodies (pAbs) were from BioSource (Camarillo, CA). Anti-Src family phospho-specific (Tyr416) and anti-Akt phospho-specific (Thr308) rabbit pAbs were from Cell Signaling Technology (Beverly, MA). Anti-Src mouse mAb (clone 327) was from Oncogene Research Products (San Diego, CA). Anti-JNK1 mouse pAb was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse mAb was from Abcam (Cambridge, MA), and anti-tubulin mouse mAb was from Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA). Mouse NMuMG cells (CRL-1636) were obtained from American Type Culture Collection (Manassas, VA). Subclones of NMuMG cells were prepared by limiting dilution in flat bottom 96-well plates. NMuMG cells and their subclones were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 4.5 g/l glucose, and 10 μg/ml insulin. Rat tail collagen type I and bovine fibronectin were from BD Biosciences PharMingen (San Diego, CA) or R&D Systems (Minneapolis, MN), respectively. For preparation of ECM-coated substrates, 100-mm dishes or 22-mm coverslips were incubated overnight at 4°C with collagen I (50 μg/ml) in 0.02 N acetic acid or fibronectin (50 μg/ml) in phosphate-buffered saline (PBS). Dishes or coverslips were then washed twice with PBS, blocked with PBS containing 1% bovine serum albumin for 30 min at room temperature, and washed twice with PBS. Serum was reduced to 1% to examine signals primarily from adhesion to ECM. LY294002, anisomycin (Calbiochem, La Jolla, CA), and SP600125 (BIOMOL Research Laboratories, Plymouth Meeting, PA) were added at the indicated concentrations. For blocking TGFβ activity, nonspecific IgG was from Zymed Laboratories, and pan-specific TGFβ rabbit pAb was from R&D Systems. For neutralization of TGFβ bioactivity, antibody was added to the medium at 10 μg/ml.

Detergent Extraction, SDS-PAGE, and Immunoblots

Monolayers of cultured cells were washed with ice-cold PBS and extracted on ice with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 0.2 U/ml aprotinin). In some experiments, protein was extracted with RIPA buffer containing 2 mM orthovanadate and 20 μM calyculin A. Extracts were centrifuged at 20,000 $\times$ *g* for 15 min at 4°C, and the supernatant was collected. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules CA). Cell extracts were resolved by SDS-PAGE (Laemmli, 1970), immunoblotted as described previously (Johnson *et al*., 1993), and quantified by densitometry using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Cell Surface Biotinylation

Cells were grown to subconfluence in 10-cm dishes, washed with PBS, and incubated with the nonmembrane-permeable biotinylation reagent sulfo-N-hydroxysuccinimidobiotin (Pierce Chemical, Rockford, IL) for 20 min on ice. After quenching with media containing 10% FBS and washing with PBS, the cells were lysed directly on the dish with RIPA buffer. Then, 500 μg protein was incubated with anti-E-cadherin (4A2) for 1 h at 4°C with rotation. Antibody affinity gel (goat affinity-purified antibody to mouse IgG; MP Biomedicals, Aurora, OH) was added to the extracts and incubated overnight at 4°C with rotation. The gel was washed three times with lysis buffer, mixed with loading sample buffer, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with horseradish peroxidase-labeled streptavidin (Pierce Chemical).

Cell Fractionation

Cells were grown to subconfluence in 10-cm dishes and lysed for 10 min on ice with gentle rocking in 1% Triton X-100, 50 mM Tris, pH 7.6, 150 mM NaCl, and 2 mM EDTA with protease inhibitors. The lysis buffer removed from the cells constituted the Triton X-100–soluble fraction. The remaining cells were lysed with sonication in SDS buffer (50 mM Tris, pH 6.8, 10% glycerol, and 2% SDS) to produce the insoluble fraction. An equal percentage of each fraction was resolved by SDS-PAGE.

Pull-Down Assays

Pull-down assays were performed as described previously (Johnson *et al*., 2004). Briefly, cultured cells were rinsed twice with cold PBS and scraped in lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF). The mixture was incubated on ice for 10 min and clarified by centrifugation. Then, 1 mg of protein was incubated with glutathione *S*-transferase (GST)-CRIB (Rac1) or GST-WASP (Cdc42) beads (Johnson *et al*., 2004) for 1 h at 4°C and centrifuged. The beads were washed three times with lysis buffer and resuspended in 2$\times$ Laemmli sample buffer for SDS-PAGE (Laemmli, 1970).

Immunofluorescence Microscopy

Cells were fixed with HistoChoice tissue fixative (Amresco, Solon, OH) and processed as described previously (Kim *et al*., 2000). When staining with phalloidin, cells were fixed in 3.7% formaldehyde for 15 min, and permeabilized with 0.2% Triton X-100 in PBS for 15 min. Cells were examined on an Axiovert 200M microscope (Carl Zeiss, Gottingen, Germany) equipped with an ORCA-ER digital camera (Hamamatsu, Houston, TX). Images were collected and processed using OpenLab software (Improvision, Boston, MA) or SlideBook software (Intelligent Imaging Innovations, Santa Monica, CA).

Conventional Reverse Transcription (RT)-PCR

Total RNA was extracted with TRI reagent and analyzed by RT-PCR using a Titanium One-Step RT-PCR kit (Clontech, Mountain View, CA) and previously reported forward and reverse primers for mouse E-cadherin (Tegoshi *et al*., 2000), mouse N-cadherin (Chung *et al*., 1998), mouse TGFβ (Derynck *et al*., 1986), and mouse GAPDH (Xu *et al*., 2000). The conditions for PCR were as follows: 94°C for 45 s, 60°C for 30 s, and 72°C for 90 s for 35 cycles for N-cadherin and TGFβ or 30 cycles for E-cadherin and GAPDH. PCR products were analyzed by electrophoresis on 1.5% agarose gels.

Quantitative Real-Time RT-PCR

Total RNA was analyzed in an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) using the following PCR protocol: 50°C for 30 min and 95°C for

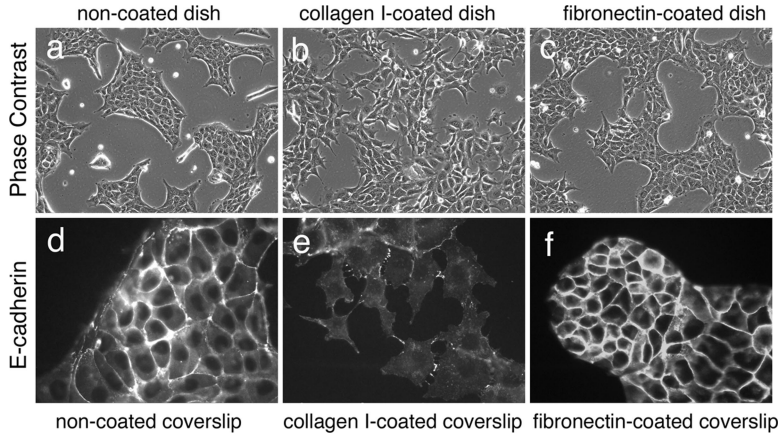

Figure 1. NMuMG/E10 cells undergo a morphological change in response to collagen I. NMuMG/E10 cells were cultured on noncoated (a and d), collagen I-coated (b and e), or fibronectin-coated (c and f) dishes (a–c) or coverslips (d–f). a–c, phase contrast pictures. d–f, immunofluorescence staining for E-cadherin (ECCD2). Photographs (a–c) were taken using a 10× objective. Photographs (d–f) were taken using a 40× oil objective.

10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Combinations of primers and probes for E-cadherin, N-cadherin, and 18S rRNA (control) were purchased from Applied Biosystems (Foster City, CA), and a reaction mixture was made using Brilliant Probe-Based QRT-PCR reagents (Stratagene) according to the manufacturer's protocol. Reactions were performed in duplicate and repeated three times, except for the real time PCR shown in Figure 3E, which was repeated two times.

Constructs, Transfections, and Infection

N-terminal green fluorescent protein (GFP)-tagged Rac1N17 and RacV12, kind gifts of Dr. Klaus Hahn (University of North Carolina, Raleigh, NC), and C-terminal myc-tagged human N-cadherin (Kim *et al*., 2000) were subcloned into LZRS-MS-Neo (Ireton *et al*., 2002). The N-cadherin short hairpin RNA (shRNA) construct in pSuperRetroPuro and the Src dominant negative construct have been described previously (Maeda *et al*., 2005). shRNA targets for integrin β1, as suggested by CODEX, were GGAAGGAAATCTTAGCTTT (nucleotides 3134–3152 of integrin β1 GenBank accession no. NM010578.1). cDNA for mouse Smad7 was generated using RT-PCR and total RNA from NMuMG/E9 cells. Phoenix 293 cells were transfected using a calcium phosphate transfection kit (Stratagene) to produce retroviral particles. Conditioned medium containing recombinant retrovirus and supplemented with 4 μg/ml polybrene was added to cells as described previously (Johnson *et al*., 2004; Maeda *et al*., 2005). Transfected or infected cells were selected with 1 mg/ml G418 (Cellgro; Mediatech, Herndon, VA) or 4 μg/ml puromycin. N-cadherin and integrin β1 knockdown and control enhanced GFP shRNA was performed as described previously (Maeda *et al*., 2005).

Transwell Motility Assays

Cells (5×10^5) were plated in the top chamber of polyethylene terephthalate membranes (BD BioCoat control culture inserts, six-well plates, pore size 8 μm; BD Biosciences, San Jose, CA). Both upper and lower sides of the inserts were coated with collagen I or fibronectin. DMEM, 1% FBS was added to the top chambers, and DMEM, 10% FBS was added to the bottom chamber. For experiments using inhibitors, dimethyl sulfoxide (DMSO), LY294002 (10 μM), or SP600125 (10 μM) were added to both the top and bottom chambers. Cells were incubated on the membranes for 4 h, and cells that did not migrate through the membrane were removed with a cotton swab. Cells traversing the membrane were stained using a HEMA3 stain set and five random fields of view at 100× magnification were counted and expressed as the average number of cells per field of view. Three independent experiments were performed, and the data are represented as the average with SD.

Three-dimensional (3D) Culture in Collagen Gels

Collagen gel cultures were performed as described previously (Montesano *et al*., 1991). In brief, 50,000 cells were resuspended in 2 ml of cold collagen solution (1 mg/ml final concentration) in a six-well dish. After the collagen solution had gelled, 2 ml of complete medium was added to each well and changed every 3 d. For inhibitory experiments, LY294002 or SP600125 was added to the complete medium. Quantification of branching was performed by counting all identifiable branch points in each colony as described previously (Soriano *et al*., 1995).

Statistical Analysis

Statistical analysis was performed using Mann–Whitney *U*- and Kruskal–Wallis tests (StatView version 5.0; Abacus Concepts, Berkeley, CA), with $p < 0.05$ considered statistically significant.

RESULTS

NMuMG/E10 Cells Undergo Collagen I-mediated Morphological Changes and N-cadherin Up-Regulation

We previously showed that NMuMG cells purchased from American Type Culture Collection are heterogeneous with respect to cadherin expression. Thus, we generated a number of subclones that express high levels of E-cadherin and showed that these clones undergo EMT in response to TGFβ (Maeda *et al*., 2005) as reported by others for the parental cells (Miettinen *et al*., 1994; Piek *et al*., 1999; Bakin *et al*., 2000; Bhowmick *et al*., 2001). For all the experiments presented here, we used clone NMuMG/E10. Our goal in this study was to determine whether NMuMG/E10 cells undergo EMT-like changes in response to various ECM molecules and to understand the mechanisms that promote ECM-induced cellular changes.

When NMuMG/E10 cells were cultured on noncoated dishes or fibronectin-coated dishes, they grew as compact colonies, had well-organized cell–cell junctions (Figure 1, a and c), and E-cadherin was localized at cell–cell borders (Figure 1, d and f). In contrast, when cultured on collagen I, NMuMG/E10 cells seemed fibroblastic and did not form compact epithelial colonies, but rather, they were scattered, as is typical for cells undergoing EMT (Figure 1b). In addition, E-cadherin staining was reduced at cell–cell borders (Figure 1e). To ensure that clone NMuMG/E10 was not unique in its response to ECM, we plated the bulk population of parental cells on noncoated, collagen I-coated, or fibronectin-coated dishes and showed that they responded in a manner similar to the clone. These data are presented as Supplemental Figure S1.

Staining for F-actin and paxillin showed that NMuMG/E10 cells were converted to a fibroblastic phenotype with well-organized stress fibers and a peripheral distribution of focal adhesions when cultured on collagen I (Figure 2, b and e). Moreover, the protein expression levels of the mesenchymal markers N-cadherin and fibronectin were up-regulated when NMuMG/E10 cells were cultured on collagen I-coated dishes for 48 h, compared with cells cultured on noncoated dishes or fibronectin-coated dishes (Figure 3, A and B). Conventional RT-PCR (Figure 3C) and quantitative real-time PCR (Figure 3D) showed that N-cadherin mRNA was up-regulated when the cells were cultured on collagen I. To determine whether N-cadherin up-regulation was an early event during collagen I–induced cellular changes, we used quantitative real-time PCR to compare N-cadherin mRNA levels at early time points in cells plated on collagen I-coated

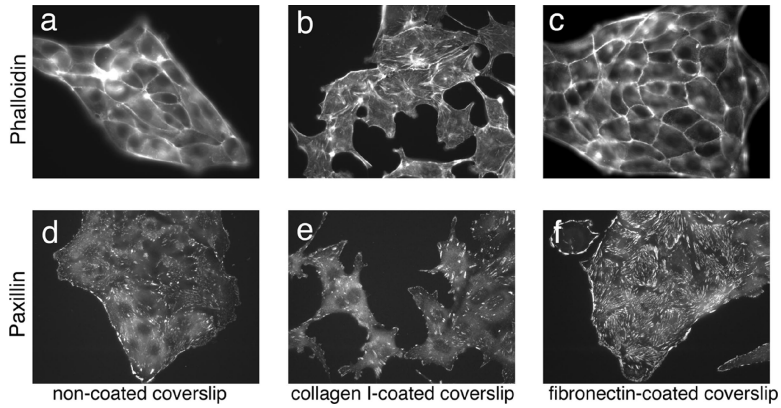

Figure 2. Collagen I–induced changes in NMuMG/E10 cells. NMuMG/E10 cells were cultured on noncoated (a and d), collagen I-coated (b and e), or fibronectin-coated (c and f) coverslips. a–c, phalloidin staining. d–f, paxillin staining. Photographs were taken using a 40× oil objective.

dishes to that of cells plated on noncoated dishes and showed that N-cadherin mRNA levels peaked at 12 h (Figure 3E).

An important aspect of EMT is down-regulation of E-cadherin activity in addition to up-regulation of N-cadherin. Because Figure 1 shows that NMuMG/E10 cells plated on collagen I for 48 h have decreased localization of E-cadherin at cell borders, even though there is no evidence for decreased expression of E-cadherin (Figure 3, A and B), we investigated the expression, solubility, and surface localization of E-cadherin in cells plated on collagen I over a longer time course. Figure 4A shows that by 3 d there was slightly less E-cadherin expressed by cells plated on collagen I-coated dishes than by cells plated on noncoated dishes. Furthermore, N-cadherin expression remained higher in cells plated on collagen I-coated dishes than in cells plated on noncoated dishes for up to 4 d. To compare the surface localization of E-cadherin, we biotin labeled the proteins on the cell surface, immunoprecipitated E-cadherin, and blotted with peroxidase-labeled streptavidin (Figure 4B). In agreement with the immunofluorescence localization shown in Figure 1, there was less surface E-cadherin in cells plated on collagen I-coated dishes than in cells plated on noncoated dishes, and this trend held for the entire 4-d time course. One measure of the extent of junctional localization of E-cadherin is to compare Triton-soluble to Triton-insoluble E-cadherin. When we gently extracted cells with Triton X-100 to remove the Triton-soluble fraction, and then extracted the remaining E-cadherin with SDS, cells plated on collagen I-coated dishes consistently had less insoluble E-

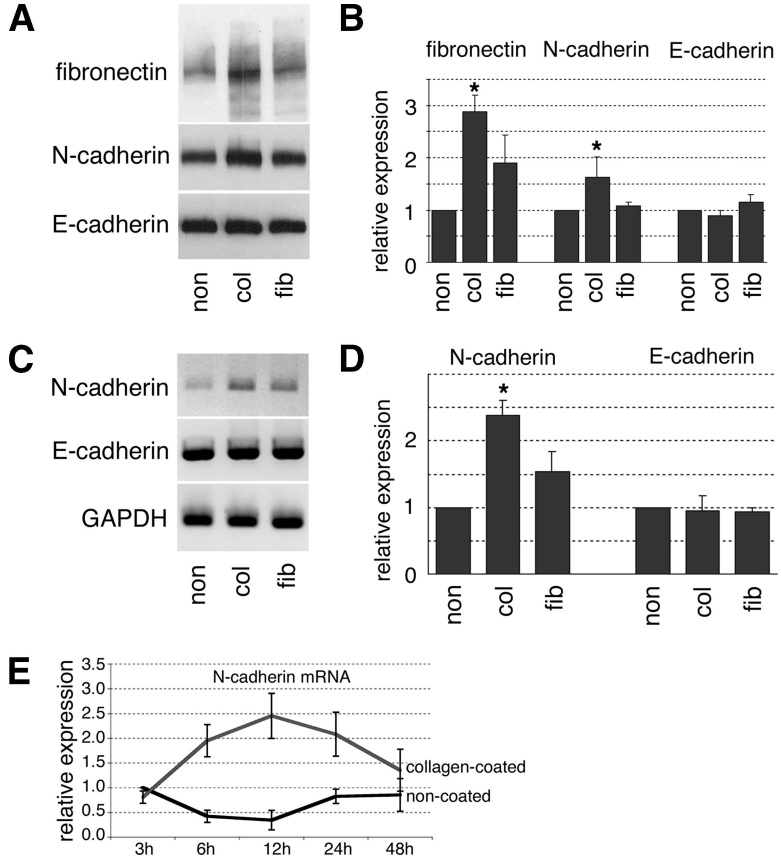

Figure 3. Collagen I–induced N-cadherin up-regulation in NMuMG/E10 cells. (A) NMuMG/E10 cells were cultured on noncoated (non), collagen I-coated (col), or fibronectin-coated (fib) dishes. Two days after seeding cells were extracted and 30 μg of protein resolved by SDS-PAGE and immunoblotted for fibronectin, N-cadherin, and E-cadherin. (B) Immunoblots were quantified by densitometry using Adobe Photoshop and normalized to GAPDH. The columns represent mean values of three independent experiments and the bars represent the SD (*$p < 0.05$; col versus non or fib). (C) RT-PCR was done for N-cadherin, E-cadherin, and GAPDH (as a control). (D) N-cadherin and E-cadherin mRNA levels were analyzed by quantitative real-time PCR. 18S rRNA was used as an endogenous control, and quantification of mRNA levels was performed in duplicate and repeated three times. The columns represent mean values and the bars represent the SD (*$p < 0.05$; col versus non or fib). (E) N-cadherin mRNA was analyzed by quantitative real-time PCR from 3- to 48-h time points. Quantification was performed in duplicate and repeated two times.

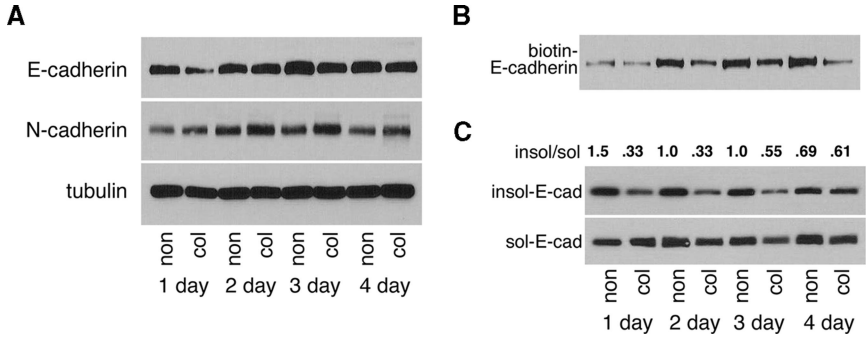

Figure 4. Collagen I–induced changes in cadherin expression in NMuMG/E10 cells. (A) NMuMG/E10 cells were cultured on noncoated (non) or collagen I-coated (col) dishes. One day to 4 d after seeding, cells were extracted, and 30 μg of protein was resolved by SDS-PAGE and immunoblotted for N-cadherin, E-cadherin, and tubulin (as a loading control). (B) Cell surface E-cadherin expression in NMuMG/E10 cells. NMuMG/E10 cells were cultured on noncoated (non) or collagen I-coated (col) dishes. One day to 4 d after seeding, cells were surface biotinylated, and 500 μg of protein was immunoprecipitated with anti-E-cadherin (4A2), resolved by SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase-labeled streptavidin. (C) E-cadherin extractability. NMuMG/E10 cells were cultured on noncoated (non) or collagen I-coated (col) dishes for 1–4 d. The soluble fraction (sol-E-cad) was extracted with 1% Triton X-100 for 10 min at 4°C with gentle rocking. The insoluble fraction (insol-E-cad) was extracted using an equal volume of SDS sample buffer. An equivalent volume of each fraction was resolved by SDS-PAGE and immunoblotted for E-cadherin. The ratio of insoluble E-cadherin to soluble E-cadherin (insol/sol) was calculated.

cadherin than cells plated on noncoated dishes (Figure 4C), suggesting these cells had fewer or smaller junctions.

To rule out the possibility that plating NMuMG/E10 cells on collagen I up-regulated the expression of TGFβ and collagen I–induced changes were actually due to autocrine TGFβ signaling, we showed that mRNA for TGFβ was not increased when cells were plated on collagen I (Supplemental Figure S2A). In addition, we showed that pan neutralizing antibodies against TGFβ did not abolish the effect of plating cells on collagen I (Supplemental Figure S2B). Further evidence that TGFβ is not a factor in collagen I–induced cellular changes comes from the fact that NMuMG/E10 cells plated on collagen I did not show nuclear translocation of Smad2/3 (Supplemental Figure S2C), a hallmark of signaling initiated by all TGFβ family members (reviewed in Shi and Massague, 2003; ten Dijke and Hill, 2004).

We have previously shown that NMuMG cells induced to undergo EMT by treatment with TGFβ down-regulate E-cadherin expression concomitant with up-regulation of N-cadherin. However, in ECM-induced changes in these cells, the E-cadherin protein and mRNA levels were the same after 2 d in cells plated on collagen I-coated dishes as in cells plated on noncoated dishes or fibronectin-coated dishes (Figure 3) and was only slightly reduced after 3 d (Figure 4). Previous studies have shown that expression of the inhibitory Smad, Smad7, prevents TGFβ-induced EMT (reviewed in Shi and Massague, 2003; ten Dijke and Hill, 2004); however, expression of Smad7 did not prevent collagen I–induced morphological changes in NMuMG/E10 cells (Supplemental Figure S2D). Thus, the data presented in Figures 1–4 show that NMuMG/E10 cells undergo changes in morphology and cadherin expression in response to collagen I that partially resembles classical EMT and that the response differs from the response of these same cells to TGFβ, suggesting that signaling pathways involved in ECM-induced cellular changes differ from those involved in TGFβ-induced EMT.

Integrin/Rac1 Signaling Promotes Collagen I–induced Cell Scattering and Up-Regulation of N-Cadherin

To confirm that integrin engagement is a component of the collagen I-mediated changes in cellular phenotype described above, we investigated the activation state of molecules known to be downstream of integrins by examining phosphorylation of FAK and paxillin. FAK is a protein tyrosine kinase that links integrin receptors to intracellular signaling pathways and is itself phosphorylated when activated (Schlaepfer *et al.*, 1999). Figure 5A shows that FAK was more highly phosphorylated when NMuMG/E10 cells were plated on collagen I-coated dishes than when they were plated on noncoated dishes or fibronectin-coated dishes. In addition, paxillin, which is downstream of integrin signaling was also more highly phosphorylated in cells cultured on collagen I-coated plates (Figure 5B). To directly investigate the role of integrin in N-cadherin up-regulation and cell scattering in response to collagen I, we used shRNA to knockdown integrin β1 expression in NMuMG/E10 cells and compared the morphology of cells plated on noncoated versus collagen I-coated dishes (Figure 5C). The morphology of the cells did not change when plated on noncoated dishes (Figure 5C, compare c with a). There was a morphological change in the integrin β1-knocked down cells when plated on collagen I-coated dishes versus noncoated dishes (Figure 5C, compare d with c); however, the integrin β1-knocked down cells were less scattered on collagen I than were the controls (Figure 5C, compare d with b). Figure 5D shows that integrin β1 was very effectively knocked down by the shRNA (top) and that knocking down integrin β1 prevented the up-regulation of N-cadherin when the cells are plated on collagen I (middle). These data further implicate integrin β1 in collagen I–induced cell scattering and N-cadherin up-regulation.

To identify potential signaling pathways that are downstream of integrins, we first asked whether Rac1 was involved, because it has been reported to regulate collagen I-dependent cell migration (Keely *et al.*, 1997; Sander *et al.*, 1998; Valles *et al.*, 2004). Increased levels of activated Rac were observed within 3 h of plating NMuMG/E10 cells on collagen I compared with cells plated on noncoated dishes (Figure 6, A and B). The activation state remained slightly higher for up to 12 h. To further investigate the role of Rac1 in collagen I–induced cellular changes, dominant-negative Rac1 (RacN17) and constitutively active Rac1 (Rac V12) were expressed in the cells. Both RacV12 and RacN17 were fused to GFP and thus migrated at a significantly higher molecular weight than the endogenous Rac1 (Figure 6C). Pull-down assays showed that activation of endogenous Rac1 was greater in control mock-transfected cells when plated on collagen I-coated dishes compared with noncoated dishes (Figure 6C, compare lane 2 with lane 1; *). Cells expressing dominant negative RacN17 showed reduced collagen I-stimulated activation of Rac1 (Figure 6C, compare lanes 3 and 4

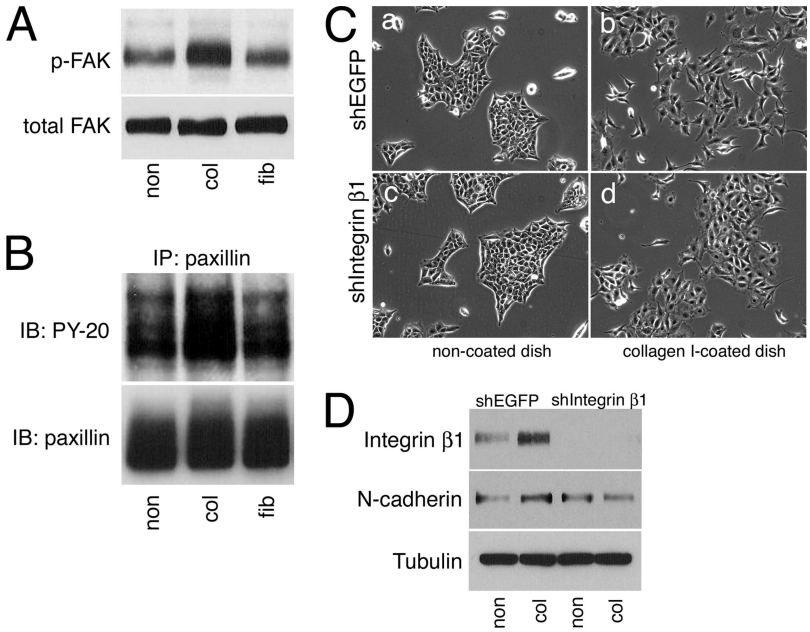

Figure 5. Involvement of integrin related molecules in response to collagen I. (A) NMuMG/E10 cells were extracted 3 h after plating on noncoated, collagen I-coated, or fibronectin-coated dishes. RIPA extracts were immunoblotted for phospho-FAK (Tyr 577) and total FAK. (B) Lysates (300 μg of protein) were immunoprecipitated with anti-paxillin mAb and resolved by SDS-PAGE. Phospho-tyrosine and total paxillin were detected by PY20 mAb and paxillin mAb immunoblots, respectively. (C) NMuMG/E10 cells infected with shEGFP (a and b) or shIntegrin β1 (c and d) were cultured on noncoated (a and c) or collagen I-coated (b and d) dishes. Two days after seeding, phase pictures were taken using a 10× objective. (D) Two days after seeding on noncoated (non) and collagen I-coated (col) dishes, cells were extracted, and 30 μg of protein was resolved by SDS-PAGE and immunoblotted for integrin β1, N-cadherin, and tubulin.

with lanes 1 and 2). The exogenous constitutively active RacV12 was bound to GTP, indicating it was active, under both plating conditions (Figure 6C, lanes 5 and 6; *).

We used phosphorylation of JNK as a measure of downstream signaling from activated Rac1. Figure 6D shows that control mock-transfected cells had higher levels of phospho-

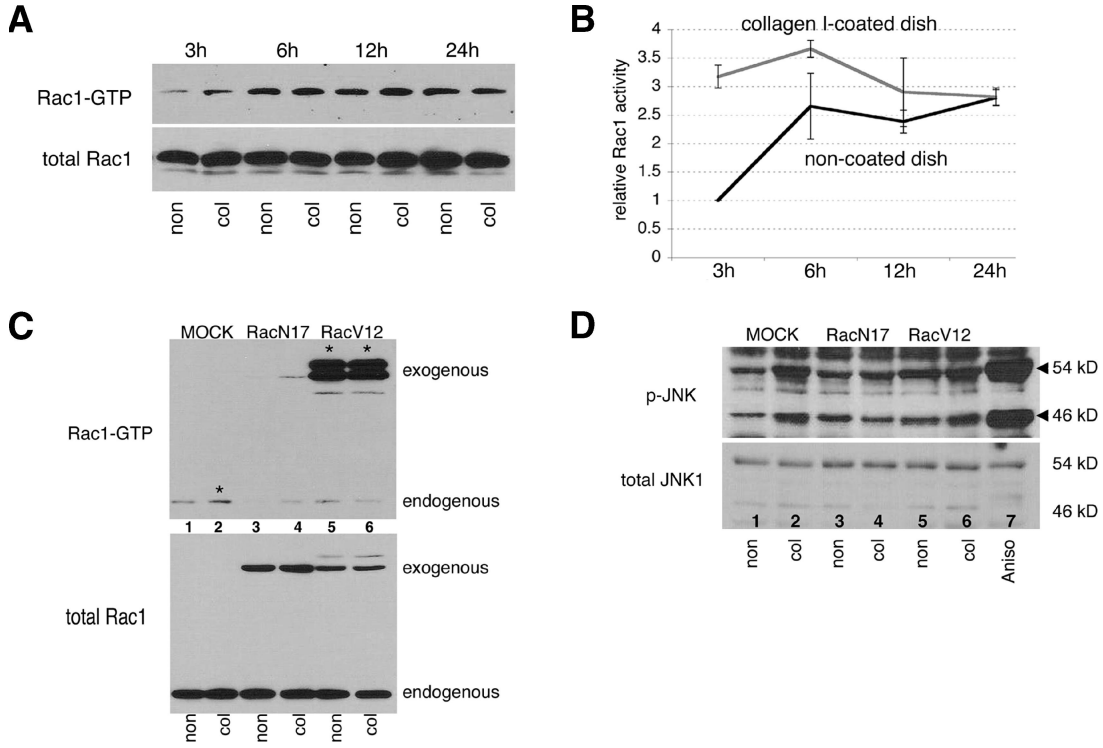

Figure 6. Rac1 is activated when cells are plated on collagen I. (A) NMuMG/E10 cells were extracted 3 to 24 h after plating on noncoated or collagen I-coated dishes. Lysates (1 mg of protein) were incubated with GST-CRIB–coupled beads and resolved by SDS-PAGE. Rac1-GTP and total Rac1 were detected by Rac1 immunoblots. (B) Rac1-GTP was quantified and normalized to total Rac1. Each pull-down experiment was repeated two times. (C) NMuMG/E10 cells were infected with retrovirus encoding the neomycin-resistance gene (MOCK), RacN17-GFP, or RacV12-GFP. Three hours after seeding on noncoated or collagen I-coated dishes, protein was extracted and pull-down assays performed as in A. (D) RIPA extracts of NMuMG/E10 cells expressing the neomycin-resistance gene (MOCK), RacN17-GFP, or RacV12-GFP were immunoblotted for phospho-JNK (p-JNK; Thr183/Thy185) and total JNK1. Cells treated with anisomycin (Aniso; 1 μg/ml) to activate JNK were used as a control to indicate the phosphorylated forms of JNK (lane 7).

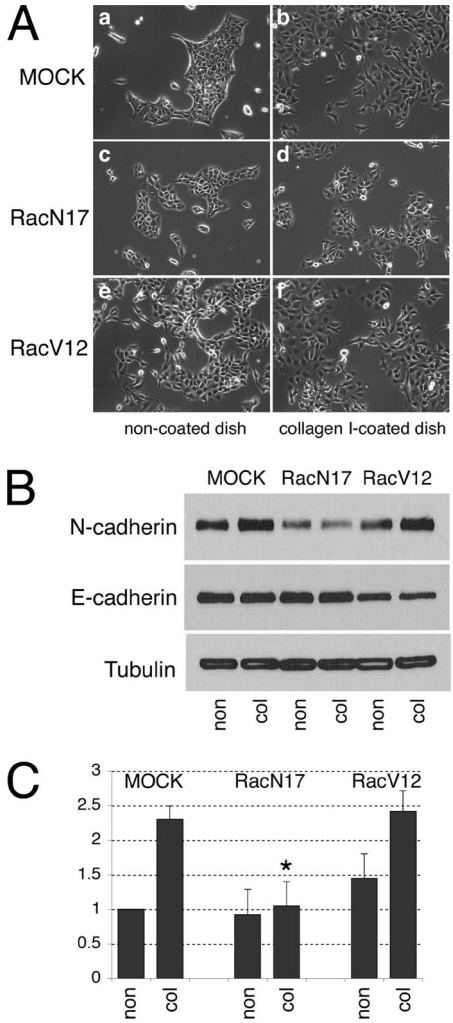

Figure 7. RacN17 inhibits collagen I–induced morphological changes and N-cadherin up-regulation. (A) MOCK, RacN17, and RacV12 infected NMuMG/E10 cells were cultured for 2 d on noncoated (a, c, and e) or collagen I-coated (b, d, and f) dishes and photographed using a 10× objective. (B) Extracts (30 μg of protein) from cells cultured on noncoated or collagen I-coated dishes for 2 d were resolved by SDS-PAGE and immunoblotted for N-cadherin, E-cadherin, and tubulin (loading control). (C) N-cadherin mRNA levels were analyzed by quantitative real-time PCR as in Figure 3D (*$p < 0.05$, col RacN17 versus col MOCK or col RacV12).

JNK when plated on collagen I-coated dishes than when plated on noncoated dishes (Figure 6D, compare lane 2 with lane 1). The positive control for phosphorylation of JNK was cells treated with anisomycin (Figure 6D, lane 7), and the two relevant phosphorylation bands at 54 and 46 kDa are pointed out by arrows. Each band was more highly phosphorylated in the mock-transfected cells when they were plated on collagen I-coated dishes, compared with cells plated on noncoated dishes. When cells were transfected with dominant negative RacN17, phosphorylation on JNK was not stimulated by plating on collagen I (Figure 6D, compare lane 4 with lane 3), and when cells were transfected with constitutively active Rac1V12, there was increased JNK phosphorylation under both plating conditions (Figure 6D, compare lanes 5 and 6 with lane 1). Thus, signaling downstream of Rac1, as measured by phosphorylation on JNK, was inhibited by RacN17 and activated by RacV12. Furthermore, the collagen I-mediated morphological changes in NMuMG/E10 cells were inhibited by dominant negative RacN17 (Figure 7A), and immunoblotting and RT-PCR showed that N-cadherin up-regulation was completely inhibited by RacN17 (Figure 7, B and C). Expression of constitutively active RacV12 caused a morphological change in cells plated on noncoated dishes, but it did not induce scattering (Figure 7A, e), and it did not induce up-regulation of N-cadherin expression in cells plated on noncoated dishes. Expression of RacV12 did, however, result in decreased E-cadherin expression that was somewhat collagen I dependent (Figure 7B) but had no effect on the ability of cells to scatter on collagen I or to up-regulate N-cadherin expression. Thus, Rac1 activation is necessary but not sufficient for collagen I–induced morphological changes and N-cadherin up-regulation. In contrast, Cdc42 was not activated in cells plated on collagen I (Supplemental Figure S3A), and Cdc42N17 had no effect on collagen I–induced cellular changes (Supplemental Figure S3B). These data are consistent with a signaling pathway whereby engagement of the integrin receptor for collagen I activates FAK, which leads to activation of Rac1.

PI3K and JNK Activation Are Involved in Collagen I–induced Cell Scattering and N-Cadherin Up-Regulation

It has been previously shown that integrin activation of FAK can activate Src family kinase or PI3K, both of which can signal to Rac1 (reviewed in Guo and Giancotti, 2004). To determine which pathway is important for signaling from integrin to Rac1 when cells are plated on collagen I, we asked whether inhibiting PI3K or Src family kinases would prevent cellular changes when NMuMG/E10 cells were plated on collagen I. The PI3K inhibitor LY294002 effectively prevented cell scattering in response to collagen I (Figure 8A). Importantly, inhibiting PI3K activity prevented phosphorylation of Akt, which is known to be downstream of PI3K in this pathway (Figure 8B). Inhibition of PI3K activity decreased the levels of activated Rac1 in cells plated on collagen I and prevented phosphorylation of JNK, which is downstream of Rac1 (Figure 8B). Inhibiting Src family kinases using dominant negative Src did not prevent the morphological changes in NMuMG/E10 cells when they were plated on collagen I (Supplemental Figure S4), confirming that the PI3K arm of the pathway regulates collagen I–induced changes in these cells.

To investigate signaling downstream of PI3K and Rac1, we treated cells with the JNK inhibitor SP600125. Figure 8A shows that inhibiting JNK inhibited collagen I–induced morphological changes in NMuMG/E10 cells. Importantly, immunoblots and PCR showed that collagen I-mediated N-cadherin up-regulation was also inhibited by the PI3K and JNK inhibitors (Figure 8, C and D). Thus, the data presented here firmly implicate signaling through PI3K to JNK in collagen I–induced cellular changes and show that both morphological changes and up-regulation of N-cadherin are regulated by the same pathway.

N-Cadherin Knockdown Inhibits Collagen I-mediated Cell Scattering

LY294002, RacN17, and SP600125 inhibited both collagen I-mediated morphological changes and N-cadherin up-regulation in NMuMG/E10 cells. Together, these data led us to speculate that up-regulation of N-cadherin itself may promote the morphological changes that occur when NMuMG/E10 cells are plated on collagen I and/or may be essential for these changes. To test this idea, we generated N-cadherin knockdown NMuMG/E10 cells and N-cadherin overex-

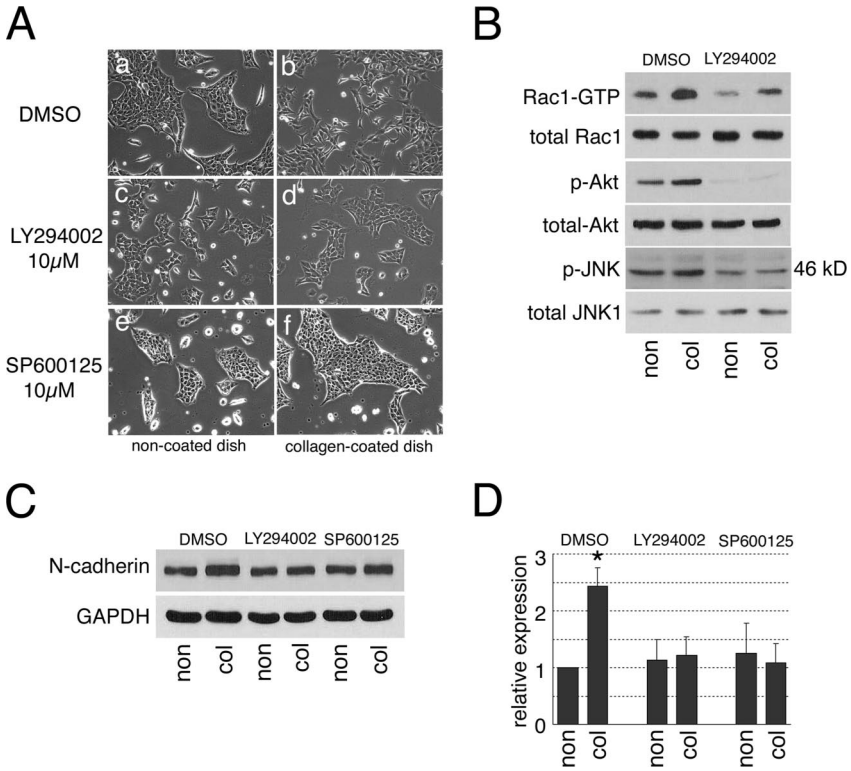

Figure 8. PI3K and JNK activities are necessary for collagen I–induced changes. (A) NMuMG/E10 cells were cultured on noncoated or collagen I-coated dishes for 2 d in the presence of the vehicle DMSO (a and b), the PI3K inhibitor LY294002 (10 μM; c and d), or the JNK inhibitor SP600125 (10 μM; e and f). Photographs were taken using a 10× objective. (B and C) RIPA extracts were made from cells cultured on noncoated or collagen I-coated dishes for 3 h in the presence of DMSO, LY294002 (10 μM; B and C), or SP600125 (10 μM; C). Pull-down assays for Rac-GTP and total Rac were performed as in Figure 6A. Immunoblots were done for phospho-Akt (Ser479), total Akt1, phospho-JNK (Thr183/Thy185) and total JNK1 (B), and with N-cadherin and GAPDH (C). (D) N-cadherin mRNA levels were analyzed by quantitative real-time PCR as in Figure 3D (*$p < 0.05$, DMSO col versus LY294002 col or SP600125 col).

pressing cells (Figure 9B). When cells were cultured on noncoated dishes, the morphology of N-cadherin knockdown and N-cadherin overexpressing cells was almost identical to that of parental NMuMG/E10 cells, in spite of significant differences in N-cadherin expression (Figure 9A). Interestingly, collagen I–induced cell scattering was inhib-

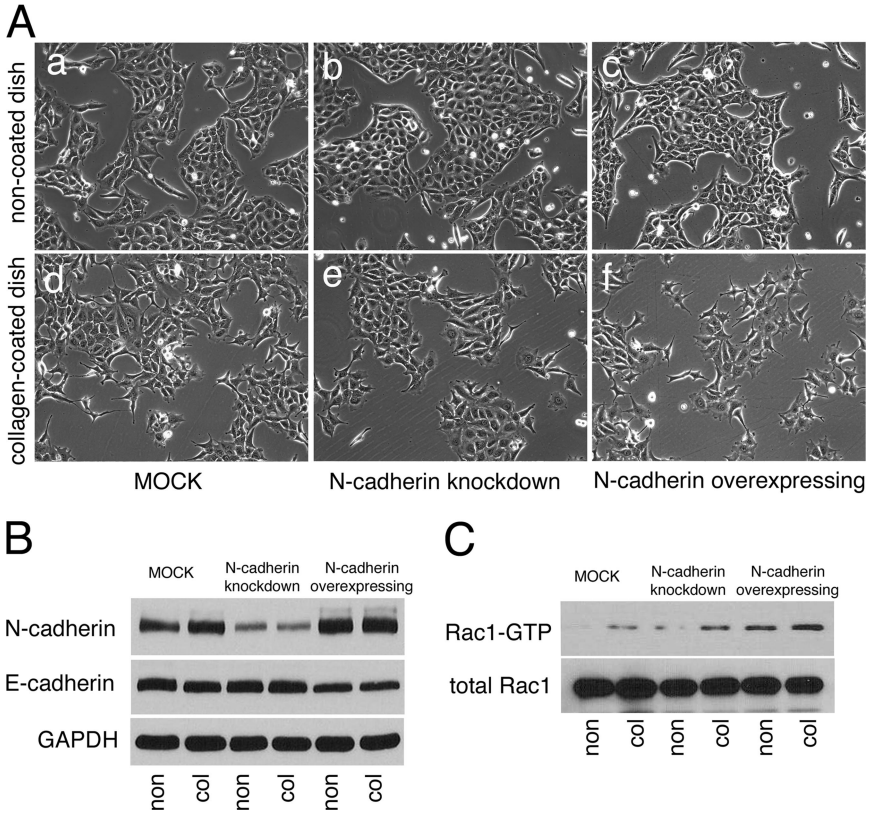

Figure 9. N-cadherin knockdown prevents collagen I–induced changes. (A) Mock-infected, N-cadherin knockdown, and N-cadherin–overexpressing NMuMG/E10 cells were cultured on noncoated (a–c) or collagen I-coated (d–f) dishes. Photographs were taken of living cells using a 10× objective. (B) RIPA extracts (30 μg of protein) from mock-infected, N-cadherin knockdown, and N-cadherin–overexpressing NMuMG/E10 were resolved by SDS-PAGE and immunoblotted for N-cadherin, E-cadherin, and GAPDH. (C) Extracts (1 mg of protein) from cells cultured on noncoated or collagen I-coated dishes for 3 h were used for pull-down assay as in Figure 6A.

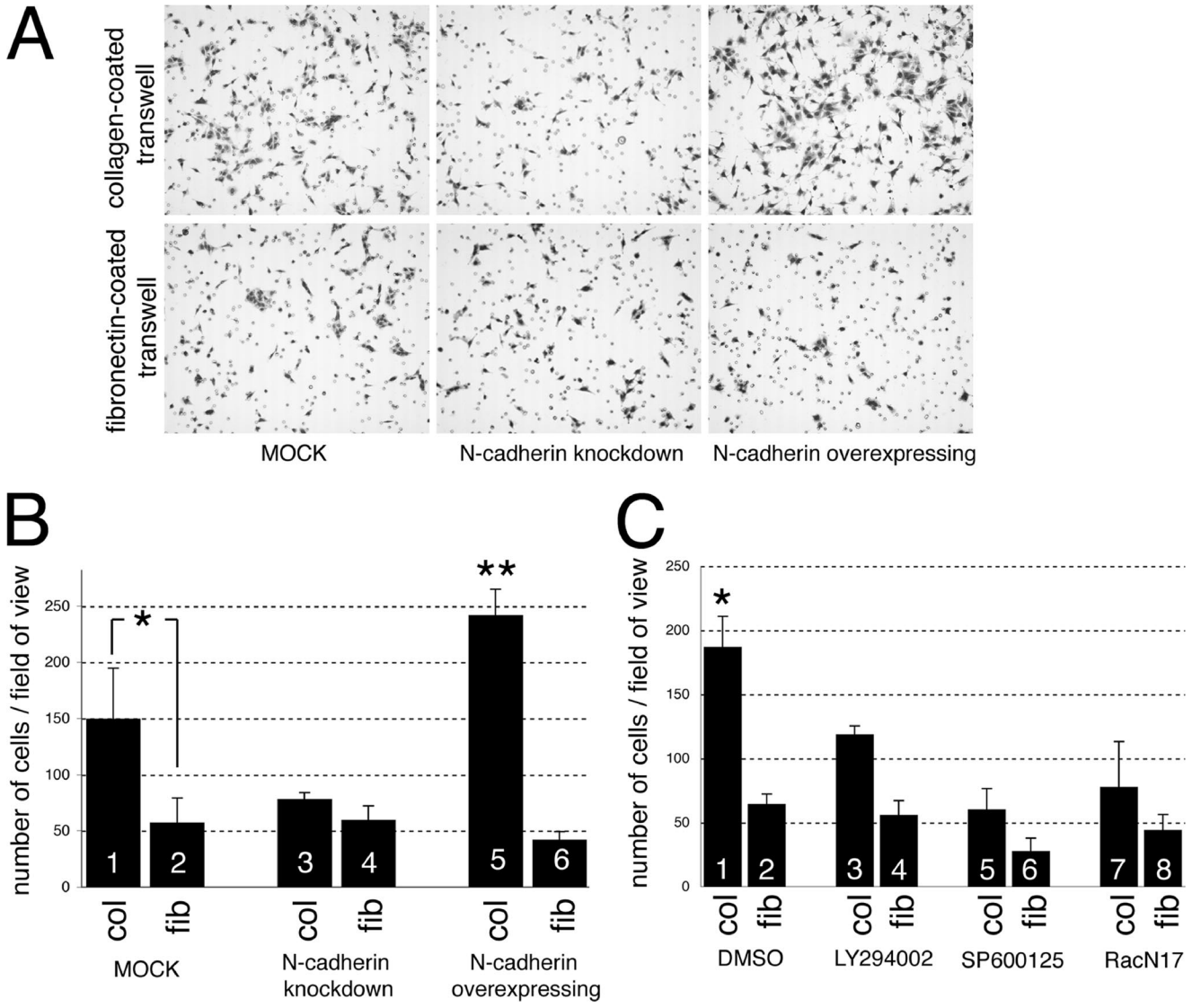

Figure 10. N-cadherin knockdown limits cell motility. (A) Mock infected, N-cadherin knockdown, and N-cadherin–overexpressing NMuMG/E10 cells were plated for 4 h on transwell filters coated in both the top and bottom sides with collagen I or fibronectin. Cells traversing the filter were photographed using a 10× objective (A) and quantified (B). The columns represent mean values and the bars represent the SD (*$p < 0.05$, 1 versus 2; **$p < 0.05$, 5 versus 1 or 3). (C) NMuMG/E10 cells were plated on coated filters, and DMSO (vehicle), LY294002 (10 μM), or SP600125 (10 μM) was added to both the top and bottom chambers. NMuMG/E10 cells expressing Rac1N17 were plated on coated filters. The columns represent mean values and the bars represent the SD (*$p < 0.05$, 1 versus 3, 5, or 7).

ited by N-cadherin knockdown (Figure 9A, e) compared with mock-infected NMuMG/E10 cells (Figure 9A, d) or N-cadherin overexpressing cells (Figure 9A, f). In fact, the scattering response of N-cadherin overexpressing cells to collagen I was greater than that of mock-transfected cells (Figure 9A, compare f with d). These data indicate that increased N-cadherin expression specifically promotes cell scattering in response to collagen I and does not promote cell scattering in the absence of collagen I, implying cells must obtain signals from collagen I in conjunction with increased N-cadherin expression to undergo scattering. Interestingly, preventing N-cadherin up-regulation in response to collagen I had no effect on the ability of the cells to activate Rac1, whereas overexpressing N-cadherin resulted in constitutive activation of Rac1, independent of the plating conditions (Figure 9C). This is consistent with previous studies from our laboratory showing that overexpression of another mesenchymal cadherin, R-cadherin, increases steady-state levels of Rac1 (Johnson *et al.*, 2004).

The Role of N-Cadherin Up-Regulation in Collagen I–induced Cell Scattering

One important aspect of epithelial to mesenchymal transitions, both during normal development and during tumorigenesis is an increase in cell motility. Thus, we asked whether plating NMuMG/E10 cells on collagen I increased cell motility. We used transwell filters coated with collagen I or fibronectin. Cells were plated in the top chamber and the cells traversing the filter in 4 h were counted. Figure 10, A and B, shows that cells plated on collagen I were 3 times as motile as cells plated on fibronectin. To determine whether collagen I–induced motility was regulated by the same

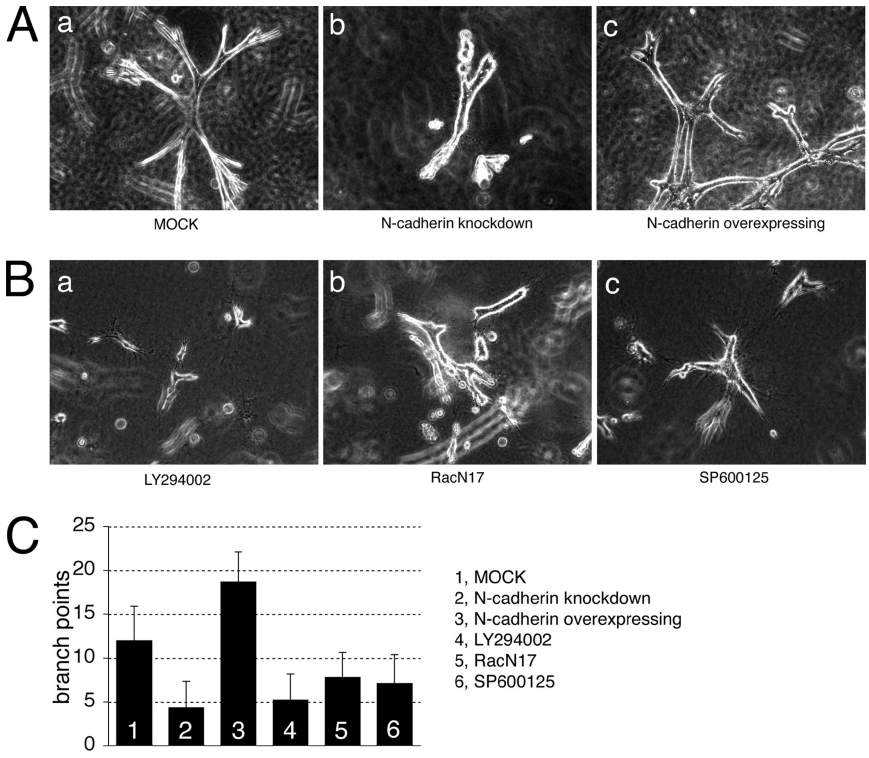

Figure 11. N-cadherin knockdown limits branching in 3D collagen gel culture. (A) 3D collagen gel cultures were performed using mock transfected, N-cadherin knockdown, and N-cadherin–overexpressing NMuMG/E10 cells. (B) 3D collagen gel cultures were performed using NMuMG/E10 cells in the presence of LY294002 (10 μM) or SP600125 (10 μM), or using NMuMG/E10 cells expressing Rac1N17. (C) Quantification of branching was performed by counting all identifiable branch points in each colony. For the experiments using inhibitors, LY294002 (10 μM) or SP600125 (10 μM) was added to the culture medium above the collagen gel. The columns represent mean values, and the bars represent the SD ($p < 0.05$; 1 versus 2 or 3, 1 versus 4, $p < 0.05$; 1 versus 5, $p = 0.11$; 1 versus 6, $p = 0.06$).

mechanisms that regulate collagen I–induced changes in cell morphology and changes in N-cadherin expression, we inhibited the activity of PI3K with LY294002, or JNK with SP600125 when plating NMuMG/E10 cells on collagen I-coated transwell filters. In addition, we inhibited Rac activity using RacN17-infected cells. In each case, the cells plated on collagen I showed significantly decreased motility compared with controls (Figure 10C). In contrast, each inhibitor had a much less profound effect on cells plated on fibronectin-coated filters, indicating that inhibition of components of the PI3K–Rac1–JNK pathway specifically inhibits collagen I–induced motility.

Some cultured mammary epithelial cell lines, including NMuMG, undergo glandular (branching) morphogenesis when cultured in 3D collagen gels (Berdichevsky *et al*., 1994; Zutter *et al*., 1999). Branching morphogenesis is an important stage in the development of a number of tissues, including the mammary gland, and branching depends on epithelial cells converting to mesenchymal cells in order to migrate. Zutter *et al*. (1999) used a variant of NMuMG cells lacking $\alpha1\beta1$ and $\alpha2\beta1$ integrins to show that the function of $\alpha2\beta1$ integrin, the major receptor for collagen I, is required for branching morphogenesis (Zutter *et al*., 1999). Because our data suggest that N-cadherin and the PI3 kinase/Rac1/JNK pathway are important players in collagen I–induced EMT-like changes in NMuMG/E10 cells, and EMT plays a role in branching morphogenesis, we asked whether expression of N-cadherin or activity of this pathway were important for NMuMG/E10 cells to undergo branching morphogenesis in collagen gels. When mock transfectants or N-cadherin overexpressing NMuMG/E10 cells were cultured in collagen gels, the cells organized into tubular and branched structures (Figure 11A, a and c), whereas N-cadherin knockdown severely retarded tube formation and branching (Figure 11A, b). Branching was quantified by counting the number of branch points (Figure 11C). Interestingly, N-cadherin overexpression enhanced the formation of tubular and branching structures, perhaps because over expression of N-cadherin enhances cell motility. Furthermore, when we inhibited PI3K with LY294002 (Figure 11B, a), JNK with SP600125 (Figure 11B, c), or Rac1 by expressing RacN17 (Figure 11B, b), the cells showed less branching than controls cells. Together, the data presented in Figures 10 and 11 indicate that N-cadherin and the PI3K–Rac1–JNK pathway play important roles in collagen I-mediated morphological changes in NMuMG cells and suggest that the PI3K–Rac1–JNK pathway is responsible for cross-talk between the collagen receptors and N-cadherin in this system.

DISCUSSION

Collagen I–induced EMT

During EMT, tightly compacted colonies of epithelial cells with strong cell–cell contact transform to scattered fibroblastic cells with minimal cell–cell contact. In addition, they convert from relatively nonmotile cells to highly motile, invasive cells. To understand how cells undergo EMT, we must first understand the pathways involved in cross-talk between integrin-mediated cell motility and cadherin-based cell–cell adhesion. Gimond *et al*. (1999) reported that expression of integrin $\beta1$ in integrin $\beta1$-null epithelial cells resulted in disruption of adherens junctions and loss of cadherin function. In addition, using an oral squamous epithelial cell line that forms multicellular aggregates when plated on nonadherent substrates, Kawano *et al*. (2001) showed that exposing cells to laminin V disrupted cadherin-mediated cell–cell adhesion and promoted cell scattering. In our studies, plating NMuMG/E10 cells on collagen I resulted in the loss of epithelial characteristics and acquisition of mesenchymal properties, such as fibroblastic morphology and increased expression of N-cadherin and fibronectin. When cells were plated on collagen I-coated coverslips, paxillin, a marker for focal contacts, was localized at the periphery of

cells compared with cells plated on fibronectin-coated coverslips, where paxillin was localized in large, mature focal adhesions that are consistent with poorly motile cells. Furthermore, a transwell migration assay showed that the motility of NMuMG/E10 cells was enhanced in collagen I-coated transwells compared with fibronectin-coated transwells. These data suggest that adhering to collagen I promotes the reorganization of cell–cell junctional complexes and induces EMT-like changes in E10/NMuMG cells, making these cells a good model for studying ECM-induced EMT.

Signaling Induced by Plating Cells on Collagen I

To identify pathways that are relevant to collagen I–induced cellular changes, we investigated signaling events known to be downstream of integrin engagement, including phosphorylation of FAK and paxillin. Each of these integrin-related molecules was more highly phosphorylated when NMuMG/E10 cells were cultured on collagen I-coated plates than when they were cultured on noncoated dishes or fibronectin-coated dishes. FAK colocalizes with integrins in focal contacts, and its phosphorylation, which is induced by integrin occupation and clustering, initiates downstream signaling events (Yamada and Miyamoto, 1995). Activated FAK is known to bind to c-Src and PI3K, leading to changes in phosphorylation of signaling molecules. Menke *et al*. (2001) reported that down-regulation of E-cadherin expression depends on Src activation when plating pancreatic cancer cell lines on collagen I. However, in our system, collagen I–induced cell scattering and up-regulation of N-cadherin were not inhibited by expression of dominant-negative Src, whereas the PI3K inhibitor LY294002 inhibited both the morphological changes and N-cadherin up-regulation. The product of PI3K activity, phosphatidylinositol 3,4,5-phosphate, is required to regulate many Rac-specific guanine nucleotide exchange factors, including Tiam1 and Vav (Han *et al*., 1998; Fleming *et al*., 2000), leading to activation of Rac. Thus, PI3K is vital to cell migration in response to integrin signaling (Shaw *et al*., 1997). Our results suggest that PI3K plays a key role in collagen I-mediated cellular changes in NMuMG/E10 cells. Bakin *et al*. (2000) reported that PI3K-Akt signaling is required for TGFβ-induced transcriptional responses, EMT, and cell migration. These authors also showed that inhibiting PI3K blocked TGFβ-mediated phosphorylation of Smad2. In our system, Smad2/3 was not translocated to the nucleus when NMuMG/E10 cells were plated on collagen I, and Smad7 overexpression did not inhibit cellular changes in response to collagen I. Thus, the signaling pathways involved in collagen I–induced cell scattering and up-regulation of N-cadherin are likely different from those activated during TGFβ-mediated EMT.

Small GTPases of the Rho family are regulators of the actin cytoskeleton (Mackay and Hall, 1998). In particular, Rac is a regulator of cell motility, and active Rac localizes at the leading edge of motile cells (Kraynov *et al*., 2000; Wojciak-Stothard *et al*., 2001). Activation of PI3K by integrin $\alpha6\beta4$ stimulates Rac-dependent migration of colon carcinoma cells (Shaw *et al*., 1997). In addition, activation of Rac and Cdc42 stimulate the motility of mammary carcinoma cells through PI3K activation (Keely *et al*., 1997). Our study provides strong evidence that Rac1 plays a crucial role as a mediator of collagen I–induced cellular changes in NMuMG/E10 cells because RacN17 inhibited the disruption of cell–cell adhesion and cell scattering. These data are consistent with a previous study showing that integrin $\beta1$ triggered the activation of Rac1, but not Cdc42 (Gimond *et al*., 1999), and suggest that Rac1, but not Cdc42, plays a major role in the regulating collagen I–induced cell scattering in NMuMG/E10 cells.

It is clear from a number of studies that integrins signal to JNK (Miyamoto *et al*., 1995; Mainiero *et al*., 1997; Oktay *et al*., 1999; Almeida *et al*., 2000). For example, Oktay *et al*. (1999) used 293 human embryonic kidney cells to show that integrin-mediated stimulation of JNK requires the association of FAK with Src and $p130^{CAS}$, the phosphorylation of $p130^{CAS}$, and the recruitment of Crk, whereas PI3K activity was not required (Oktay *et al*., 1999). In contrast, integrin-mediated activation of JNK in human keratinocytes requires Ras–PI3K–Rac signaling (Mainiero *et al*., 1997). In our studies, the PI3K inhibitor LY294002 completely inhibited Rac–JNK signaling in response to collagen I. Thus, it seems that integrin-mediated JNK activation occurs via different pathways in different cell types.

The study presented here agrees with Sander *et al*. (1998) who showed that PI3K regulates cell migration in MDCK cells in a collagen-dependent manner. In their study, Rac activation inhibited motility of MDCK cells plated on fibronectin or laminin by promoting E-cadherin cell-cell contacts, but promoted motility of MDCK cells plated on collagen by preventing E-cadherin contacts. In their system plating cells on collagen also decreased surface E-cadherin without affecting total levels of this protein. A number of studies, including the Sander study, have implicated E-cadherin as a suppressor of cell motility, whereas studies from our laboratory and others have shown that the expression of N-cadherin or other mesenchymal cadherins can promote motility, even in cells that continue to express E-cadherin. Thus, is clear that motility and cadherin function are regulated differently in different cells, and this may be one reason tumor cell invasion and motility are so difficult to decipher (reviewed in Cavallaro *et al*., 2002; Cavallaro and Christofori, 2004).

Phosphorylation of transcription factors by JNK promotes expression of matrix metalloproteinases, which implicates integrin–JNK signaling in tumor cell invasion (Davis, 2000; Schlaepfer *et al*., 2004). The current study shows that expression of N-cadherin is up-regulated by collagen I through integrin–JNK signaling. Likewise, De Wever *et al*. (2004) reported that up-regulation of N-cadherin by TGFβ occurs via JNK activation in myofibroblasts. The N-cadherin promoter is reported to have AP1 binding sites (Li *et al*., 1997; Le Mee *et al*., 2005), and Jun is a member of the activator protein-1 family of transcription factors. In our system, collagen I stimulates JNK activity, likely through integrin–Rac signaling, which leads to phosphorylation of Jun.

The Role of N-Cadherin Up-Regulation in Collagen I–induced Phenotypic Changes in NMuMG/E10 Cells

Knocking down N-cadherin in NMuMG/E10 cells interfered with collagen I-mediated morphological changes. We previously showed that knocking down N-cadherin expression did not interfere with TGFβ-mediated morphological changes in NMuMG cells (Maeda *et al*., 2005), suggesting that different signaling pathways are involved in TGFβ and collagen I–induced cellular changes in NMuMG cells. Furthermore, transwell assays showed that N-cadherin–overexpressing NMuMG/E10 cells were significantly more motile than mock-transfected or N-cadherin knockdown cells in collagen I-coated transwells but not in fibronectin-coated transwells, suggesting that N-cadherin up-regulation is associated specifically with collagen I-mediated cell motility.

Interestingly, N-cadherin knockdown also inhibited cord formation and branching of NMuMG/E10 cells in collagen gels. Soriano *et al*. (1995) reported that either conditioned me-

dium from fibroblasts or hepatocyte growth factor-stimulated cord formation by NMuMG cells. Here, we show for the first time that expression of N-cadherin is necessary for formation of these structures and for branching in collagen gels. Our findings suggest that up-regulation of N-cadherin is an important component of ECM-induced cell scattering in cancer progression and may also be important in normal mammary gland development. In conclusion, N-cadherin is up-regulated through PI3K–Rac1–JNK signaling when NMuMG/E10 cells are plated on collagen I, and N-cadherin up-regulation is necessary for increased cell motility in response to collagen I and for generation of cord structures in 3D collagen gels.

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