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Immediate and Delayed Effects of E-Cadherin Inhibition on Gene Regulation and Cell Motility in Human Epidermoid Carcinoma Cells

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The invasion suppressor protein, E-cadherin, plays a central role in epithelial cell-cell adhesion. Loss of E-cadherin expression or function in various tumors of epithelial origin is associated with a more invasive phenotype. In this study, by expressing a dominant-negative mutant of E-cadherin (Ec1WVM) in A431 cells, we demonstrated that specific inhibition of E-cadherin-dependent cell-cell adhesion led to the genetic reprogramming of tumor cells. In particular, prolonged inhibition of cell-cell adhesion activated expression of vimentin and repressed cytokeratins, suggesting that the effects of Ec1WVM can be classified as epithelial-mesenchymal transition. Both short-term and prolonged expression of Ec1WVM resulted in morphological transformation and increased cell migration though to different extents. Short-term expression of Ec1WVM up-regulated two AP-1 family members, c-*jun* and *fra-1*, but was insufficient to induce complete mesenchymal transition. AP-1 activity induced by the short-term expression of Ec1WVM was required for transcriptional up-regulation of AP-1 family members and down-regulation of two other Ec1WVM-responsive genes, *S100A4* and *igfbp-3*. Using a dominant-negative mutant of c-Jun (TAM67) and RNA interference-mediated silencing of c-Jun and Fra-1, we demonstrated that AP-1 was required for cell motility stimulated by the expression of Ec1WVM. In contrast, Ec1WVM-mediated changes in cell morphology were AP-1-independent. Our data suggest that mesenchymal transition induced by prolonged functional inhibition of E-cadherin is a slow and gradual process. At the initial step of this process, Ec1WVM triggers a positive autoregulatory mechanism that increases AP-1 activity. Activated AP-1 in turn contributes to Ec1WVM-mediated effects on gene expression and tumor cell motility. These data provide novel insight into the tumor suppressor function of E-cadherin.

E-cadherin is an epithelial calcium-binding transmembrane glycoprotein that mediates formation of adherens junctions ensuring stable homophilic cell-cell adhesion. The extracellular domain of E-cadherin consists of five cadherin repeats involved in the formation of parallel E-cadherin dimers. N termini of parallel dimers interact with other parallel dimers exposed on the membrane of neighboring cells, forming complexes in *trans*, linking epithelial cells to each other (9, 64). The structural basis of the formation of *trans* dimers is the mutual incorporation of Trp^2 into a hydrophobic pocket of the interacting E-cadherin molecule (54). Cytoplasmic E-cadherin domains interact with either β- or γ-catenin, which in turn binds α-catenin, providing a link with the actin cytoskeleton and hence strengthening adhesion (9, 16). Disruption of E-cadherin-mediated intercellular adhesion is a hallmark of epithelial-mesenchymal transition (EMT), a phenomenon which occurs at certain stages of normal development and in the malignant progression of carcinoma (59, 60). Different molecular mechanisms including gene mutations (4, 26, 66), hypermethylation of the promoter (17), and transcriptional silencing by transcriptional repressors (Snail, Slug, ZEB-2/SIP1, ZEB-1, and E12/E47) (2, 8, 6, 14, 20, 46) contribute to the inactivation of E-cadherin linked with tumor progression. Reexpression of E-cadherin may induce morphological reversion and suppress cell growth and invasion suggesting an important function for E-cadherin in EMT (24, 56, 58, 67). The mechanism of tumor suppressor function of E-cadherin is not completely understood and may be linked with its role in signal transduction. Indeed, E-cadherin has been implicated not only in epithelial adhesion but also in the regulation of cell signaling. Being an important player in the Wnt signal transduction pathway, β-catenin links E-cadherin with cellular signaling networks (9, 16, 29, 47). In different systems, sequestration of β-catenin by the cytoplasmic domain of E-cadherin prevents its nuclear translocation and inhibits β-catenin/T-cell factor (TCF)-mediated transcriptional activity (42, 51). In a model of Fos protein-induced EMT, loss of E-cadherin activated β-catenin signaling in murine nontumorigenic Ep-1 cells (19). Inhibition of β-catenin signaling by E-cadherin may result in suppression of cell growth, providing a molecular basis for adhesion-independent tumor suppression function of E-cadherin (24, 57). A direct link between the functional status of E-cadherin and β-catenin signaling has been demonstrated in colon carcinoma cells SW480 harboring a mutant APC gene. In these cells, inhibition

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of adherens junctions by an anti-E-cadherin blocking antibody resulted in activation of β-catenin/TCF-dependent transcription with subsequent activation of the transcriptional repressor, Slug, and repression of E-cadherin gene transcription (15). However, in other in vitro models of EMT, loss of E-cadherin expression did not result in increased β-catenin/TCF transcriptional activity (14; J. Mejlvang et al., unpublished data). Moreover, β-catenin/TCF transcriptional activity does not correlate with E-cadherin status in breast, gastric, and pancreatic carcinoma cell lines (7, 61). It has been suggested that E-cadherin influences cell signaling through receptor tyrosine kinases (RTKs). E-cadherin and epidermal growth factor receptor (EGFR) colocalize to basolateral areas of epithelial cells and have been reported to form multicomponent complexes (28, 44). Formation of adhesive complexes leads to transient ligand-independent activation of EGFR and subsequent activation of mitogen-activated protein kinase (MAPK) (43), phosphatidylinositol 3-kinase (30, 43) signaling cascades, and Rac1 (5, 30). E-cadherin engagement may influence the activity of small GTPases via Src-dependent phosphorylation of RhoA-specific GTPase-activating protein p190RhoGAP (39). In dense epithelial cultures, E-cadherin also activates another RTK, EphA2, and inhibits cell proliferation (68). Recently, E-cadherin-mediated adhesion has been shown to repress ligand-induced activation of several RTKs including EGFR/Neu, insulin-like growth factor 1 receptor and c-Met in Madin-Darby canine kidney (MDCK) (48) but not in SW480 cell lines (15). However, although it is documented that E-cadherin affects cell signaling through RTKs, cytoskeletal reorganization, and β-catenin/TCF, there is a substantial lack of experimental work investigating the consequence of inhibition of E-cadherin-mediated adhesion for gene regulation.

In this report, we demonstrate that prolonged functional inactivation of E-cadherin by a dominant-negative E-cadherin mutant, Ec1WVM, is sufficient to induce full EMT in A431 cells. Short-term inactivation of E-cadherin has a lesser effect on the expression of target genes but is sufficient to activate the transcription factor AP-1. Activation of AP-1 by Ec1WVM appeared to be essential for some of its transcriptional effects. In addition, Ec1WVM regulates tumor cell motility in an AP-1-dependent manner.

MATERIALS AND METHODS

Cell lines and transfections. A431, a human vulvar epidermoid adenocarcinoma cell line, and all clones were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Doxycycline (DOX; 2 μg/ml) was added for indicated time periods. For prolonged treatments, DOX-containing medium was changed every second day. All transfections of plasmid DNA were performed by electroporation with a single pulse of 250 V and 250 μF by using the Gene Pulser Xcell electroporation system (Bio-Rad). To generate clones with stable expression of Ec1WVM, A431 cells were transfected with a pCMVEc1WVM plasmid (12) provided by S. Troyanovsky (Washington University Medical School, St. Louis, Mo.), and clones with altered (clones W1 to W6) and unchanged morphology (clones NT-1 and NT-2) were selected in the presence of 200 μg/ml of G418. To generate A431 clones with inducible expression, we first transfected A431 cells with the pUHD-172.1 construct and obtained A431 clones expressing the TET-responsive transcriptional activator, rtTA. Individual clones were analyzed by transfection with the pUHC13-3 construct encoding firefly luciferase. Luciferase activity was detected in cells growing in the presence or absence of DOX for 48 h, and a clone with minimal leakage was selected (clone A431-TET-on). In the second round of transfection, A431-TET-on cells were transfected with pBI-Ec1WVM, pBI-Ec1WVM-TAM67, or pUHD-c-Fos constructs along with pTK-Hyg vector (BD Bioscience Clontech). Clones were selected in the presence of 60 μg/ml hygromycin B, and the inducibility of Ec1WVM, TAM67-green fluorescent protein (GFP), and c-Fos was examined by Western blotting and immunofluorescence analysis.

Plasmids. To generate pBI-Ec1WVM, the Ec1WVM sequence was excised from pCMVEc1WVM and subcloned into multiple cloning site I of a bidirectional tetracycline (TET)-responsive vector pBI (BD Clontech). To construct a vector with simultaneous expression of Ec1WVM and a dominant-negative AP-1 mutant, a fragment coding for the TAM67-GFP fusion protein was excised from pGFP-TAMpuro (27) (provided by R. Hennigan, University of Cincinnati, Cincinnati, Ohio) and inserted into multiple cloning site II of pBI-Ec1WVM. To generate pUHD-c-Fos, c-Fos cDNA was cut out from pCMV-c-Fos and subcloned into pUHD-10-3.

Gene reporter assays. To determine β-catenin/TCF/lymphoid enhancer factor (LEF) transcriptional activity, 31D6 cells were transfected with 2 μg of pTOPFLASH or pFOPFLASH reporter constructs. The efficiency of each transfection was monitored using 400 ng of cotransfected β-galactosidase expression vector, pCMVβ-gal (Invitrogen). Cells were maintained with or without DOX for 48 h and lysed, and the luciferase activity was measured with a Lumat LB9501 tube luminometer (Berthold). The lysates obtained were also tested for β-galactosidase activity by using *o*-nitrophenyl-β-D-galactopyranoside (Sigma) as a chromogenic substrate. Results were expressed as a ratio of pTOPFLASH and pFOPFLASH reporter activities normalized to the activity of β-galactosidase in each experiment. To examine AP-1 activity, cells were transfected with an AP-1-dependent reporter pTREx5Luc containing five copies of an AP-1-binding element upstream of the minimal c-*fos* promoter (13). To demonstrate the specificity of AP-1 activation, we used a pRSVLuc reporter (13) that is largely AP-1-independent. Transfected cells were incubated for 2 days with or without DOX, and luciferase activity was measured and normalized to the β-galactosidase activity.

One-dimensional SDS electrophoresis and Western blotting. Proteins (10 or 20 μg) were denatured, separated on precast 4 to 20% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels (Invitrogen), and then transferred to Immobilon-P membranes (Millipore) by the standard procedure. Following protein transfer, blots were incubated in blocking solution with primary antibody at a dilution of 1:1,000 (for anti-myc tag antibody, clone 9E10; Santa-Cruz Biotech), 1:2,000 (for anti-E-cadherin antibody; BD Biosciences), 1:400 (for anti-GFP antibody; BD Biosciences), or 1:500 (for anti-c-Fos, anti-Fra-1, and anti-c-Jun antibodies; Santa-Cruz Biotech). Immunoreactive proteins were detected using an enhanced chemiluminescence system (Amersham).

Metabolic labeling. Cells were grown to approximately 70% confluence in microtiter 24-well culture dishes and labeled overnight in Dulbecco's modified Eagle's medium lacking methionine and containing 1% dialyzed fetal calf serum and 1 mCi/ml [^{35}S]methionine. Following labeling, cells were gently washed twice with phosphate-buffered saline solution and harvested by solubilization in lysis buffer for two-dimensional polyacrylamide gel electrophoresis (2D PAGE).

2D PAGE and image analysis. After cells were washed, excess phosphate-buffered saline solution was removed from the wells. A total of 50 μl of lysis buffer (40) was overlaid on cell monolayers, and the cells were lysed in solution by gentle pipetting. Samples were kept at −20°C until use. Whole protein lysates were subjected to isoelectrofocusing 2D PAGE as previously described (11). From 20 to 35 μl of sample was applied to the first dimension. Proteins were visualized using autoradiography and/or phosphorimaging followed by a silver staining procedure compatible with mass spectrometry analysis (25). Image analysis was performed using PDQUEST 7.1 software (Bio-Rad). Detection of low-abundant protein spots on silver-stained gels was highly enhanced by the superimposition of the dry silver gel with the corresponding autoradiograph.

Protein identification by mass spectrometry. Protein spots of interest were excised from the dry silver-stained gels, followed by rehydration in water for 30 min at room temperature. Proteins were "in-gel" digested with bovine trypsin for 12 h as previously described (55). The reaction was stopped by adding trifluoroacetic acid up to 0.4%, followed by shaking for 20 min at room temperature to increase peptide recovery. Peptides were concentrated on microcolumns containing C_{18}-based 3M Empore plugs (49) and eluted with 50% acetonitrile–0.2% trifluoroacetic acid directly on the target and cocrystallized with cyano matrix (2 mg/ml cyano-4-hydroxycinnamic acid in 0.5% trifluoroacetic acid–acetonitrile, 1:2 [vol/vol]). The extraction procedure strongly increased the amount of peptides, thus allowing direct sequence analysis of low intensity peptides. Mass spectrometry was performed using a Reflex IV matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer equipped with a Scout 384 ion source. All spectra were obtained in positive reflector mode with delayed extraction, using an accelerating voltage of 28 kV. Each spectrum represented an average of 100 to 200 laser shots, depending on the signal-to-noise ratio. The resulting mass spectra were internally calibrated by using the autodi-

gested tryptic mass values (805.417, 906.505, 1153.574, 1433.721, 2163.057, and 2273.160) visible in all spectra. Calibrated spectra were processed by the Xmass 5.1.1 and BioTools 2.1 software packages (Bruker Daltonik, GmbH). All spectra were analyzed manually as previously described (10).

Microarray hybridization. Hybridization of Atlas human cDNA expression arrays (Clontech) was performed basically as recommended by the manufacturer. Briefly, filters were prehybridized for 12 to 16 h at 68°C in 10 ml of ExpressHyb solution plus 100 μg/ml denatured sheared salmon sperm DNA. Radiolabeled probes were purified, heat denatured, and then added to 5- to 10-ml aliquots of hybridization buffer containing salmon sperm DNA. The final probe concentration was 5×10^6 to 10×10^6 cpm/hybridization. After extensive washing (three times with 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–1% SDS and two to three times with 0.1× SSC–0.5% SDS, each for 30 min at 68°C), the membranes were subjected to phosphorimaging analysis, and differential signals were identified by AtlasImage software.

Northern blotting. For Northern blot analysis, total RNA was isolated by the guanidine isothiocyanate method and separated in 1.2% agarose gels. RNA blotting and hybridization were performed as previously described (50). Radioactive DNA probes were synthesized using a random-primed labeling kit (Amersham). For radioactive labeling, 200- to 300-bp cDNA fragments corresponding to coding parts of identified genes were generated by reverse transcriptase PCR.

RNA interference. Purified and annealed synthetic oligonucleotides were purchased from Ambion (Austin, TX). The target sequence for Fra-1 was validated previously (63). The target sequence for c-Jun was GAUCCUGAAACAGAGC AUG. A total of 2×10^6 cells were transfected with 2 μg of small interfering RNA (siRNA) by the nucleofection technique in buffer V (nucleofection protocol T-20). The nucleofector device and a nucleofection kit were obtained from Amaxa (Cologne, Germany) and used in accordance with the manufacturer's recommendations. At 30 h after transfection, cells were harvested, counted, and processed for cell motility assays or Western blotting.

Cell motility assays. For wound-healing assays, wounds were generated by 20-μl pipette tips in confluent cultures of cells growing in 6- or 24-well plates. Areas of wounds were marked and photographed at different time points using a digital camera attached to a phase-contrast microscope (Nikon TE 2000-S). Where indicated, cells were maintained in the presence of DOX for 48 h prior to the creation of wounds. A transwell migration assay was performed using 24-well transwell plates containing 8-μm-pore-size polycarbonate filters (Corning Costar Corp., Cambridge, MA). A total of 1×10^5 cells were added to the top chambers and incubated overnight. Adhered cells were allowed to migrate toward serum gradient used as a chemoattractant in the lower chamber for 4 h. Those cells that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells that migrated to the underside of transwell filters were fixed, stained with a Gurr rapid staining kit (BDH), and counted by bright-field microscopy at a magnification of ×200 in four random fields using the ImageJ program.

RESULTS

Long-term expression of a dominant-negative E-cadherin mutant in A431 cells affects gene expression. Though the involvement of E-cadherin in cell signaling is documented, the effects of E-cadherin dominant-negative mutants on gene regulation have not been systematically studied. To examine whether the prolonged inhibition of cell-cell adhesion in epithelial cells influences gene expression, we generated clones of A431 human epidermoid carcinoma cells expressing a dominant-negative E-cadherin mutant, Ec1WVM. This mutant harbors a Trp^2/Ala amino acid substitution in the first cadherin-like repeat, leading to an inability of the mutant protein to form *trans* dimers. The dominant effect of Ec1WVM on cell morphology has been described earlier (12). Ec1WVM contains a C-terminal six-myc tag epitope and a 17-amino-acid deletion in the cytosolic domain, eliminating the recognition by a commercial anti-E-cadherin antibody (clone C20820; BD Bioscience). These modifications allow differentiation between wild-type and mutant forms of E-cadherin in transfected cells. We selected six clones that exhibited altered fibroblastoid morphology (clones W1 to W6) and two clones (NT-1 and NT-2) that were morphologically indistinguishable from the parental cells (Fig. 1A). As expected, W1 to W6 but not NT-1 and NT-2 clones expressed Ec1WVM (Fig. 1B). Expression levels of the endogenous E-cadherin was significantly lower in W1 to W6 clones than in NT1, NT2, or parental cells (Fig. 1B), consistent with its destabilization in cells expressing different dominant-negative E-cadherin mutants (12, 38, 65). To test whether the expression of Ec1WVM resulted in alteration in the cellular content of other proteins, we employed a proteomic approach based on 2D PAGE coupled with MALDI-TOF mass spectrometry. Cells were [^{35}S]methionine labeled, and total protein extracts from two clones expressing Ec1WVM (W2 and W3) and two control clones (NT-1 and NT-2) were subjected to 2D gel electrophoresis. Approximately 800 protein spots were detected on average in each gel. A total of 350 well-focused and relatively abundant proteins were matched and selected for quantitation. Steady-state levels of [^{35}S]methionine incorporation were estimated as a mean value for each protein spot in all sample pairs. The levels of actin as well as the total quantity of valid spots were used to normalize the amount of labeled proteins that entered the gels. Our results showed that the majority of the 350 quantitated proteins which represented essentially the most abundant components of the A431 proteome, showed no significant alterations in their levels in cells expressing the E-cadherin mutant compared with NT-1 and NT-2 clones. However, we identified 10 proteins (or 2.8% of the proteome) that were consistently deregulated by a factor of 2.0 and more. The identity of seven deregulated proteins was determined by MALDI-TOF mass spectrometry. The most striking up-regulation was observed for the mesenchymal marker vimentin (by a factor of 100) (Fig. 1C). As can also be seen from Table 1 and Fig. 1C, three keratins, namely, keratin 15 and two isoforms of keratin 13, were highly down-regulated in A431 cells expressing Ec1WVM.

As an independent approach to examine Ec1WVM-dependent gene expression and to test whether gene transcription was affected, we employed BD Atlas human general cDNA and human cancer cDNA expression arrays containing in combination approximately 900 spotted genes. To minimize the effects of clonal variations, cDNA from two Ec1WVM-positive (W1 and W3) and two control clones (NT-1 and NT-2) was applied. Using Atlas arrays, we have identified nine genes (or 1% of all spotted genes) differentially expressed in epithelial clones versus clones with compromised intercellular adhesion. Two genes (keratin 13 and vimentin) identified in 2D protein gels were present on Atlas membranes and demonstrated differential transcription in Ec1WVM-expressing versus control clones. Six of the genes identified by Atlas arrays exhibited enhanced expression in A431 clones with compromised adhesion, whereas transcription of three genes was inhibited. Validation of the results obtained from the analysis of the cDNA array was performed by Northern blotting (Fig. 2). The effects of Ec1WVM on gene expression varied from subtle activation of urokinase plasminogen activator (1.7-fold activation) to the initiation of de novo transcription of vimentin. The genes with altered expression (summarized in Table 1) play a role in epithelial-mesenchymal transition/tumor cell invasion (six genes), signal transduction (six genes), gene regulation (two genes), or metabolism (two genes). Of importance, activation

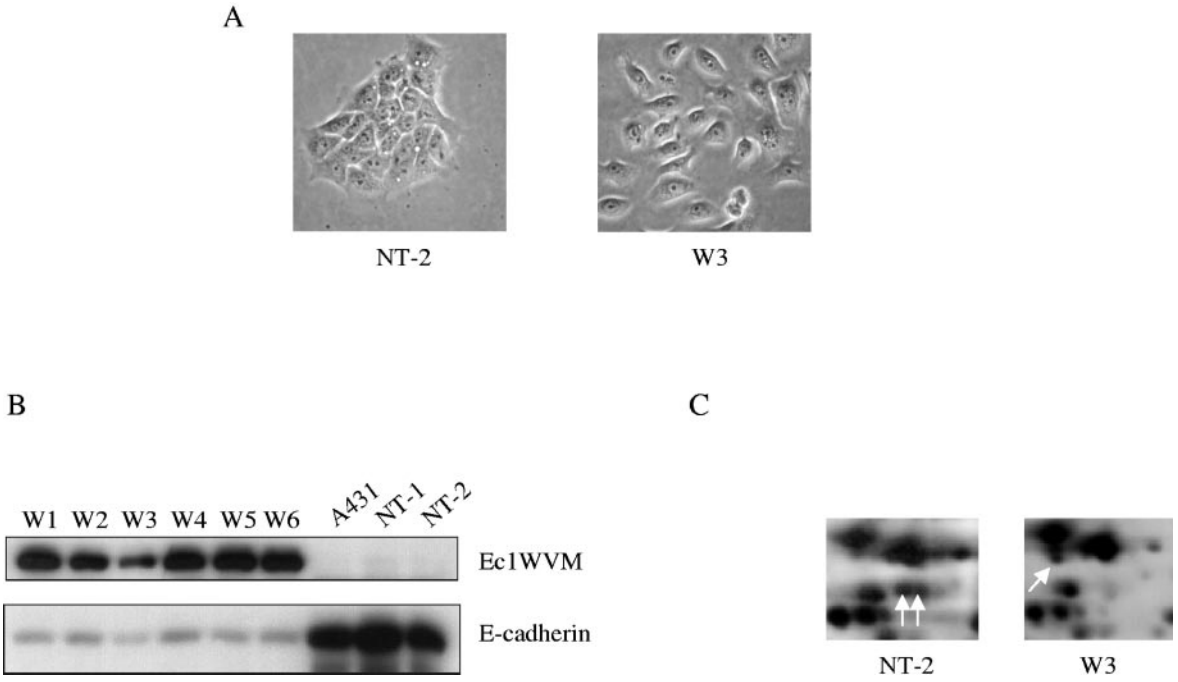

FIG. 1. Characterization of stable A431 clones expressing Ec1WVM. (A) Phase-contrast images of NT-2 and W3 clones. (B) Detection of wild-type E-cadherin and Ec1WVM in clones with altered (W1 to W6) and epithelial morphology (NT-1 and NT-2). A total of 20 μg of proteins was analyzed by Western blotting with antibodies as indicated. (C) 2D gel (isoelectrofocusing) autoradiographs of [^{35}S]methionine-labeled proteins from NT-2 and W3 cells. Only fractions of 2D gel autoradiographs are shown. The positions of keratin 13, keratin 13 variant (NT-2 panel), and vimentin (W3 panel) are indicated by arrows.

TABLE 1. Genes and proteins up- or down-regulated in A431 clones expressing Ec1WVM: summary of cDNA array analysis and 2D PAGE combined with mass spectrometry data

Gene and function[a]	Effect of Ec1WVM[b]	Method of detection[c]
Epithelial mesenchymal transition/ tumor cell invasion		
Vimentin	+	Array and 2D
Cytokeratin 13	−	Array and 2D
Cytokeratin 15	−	2D
S100A4	−	Array
MMP-2	+	Array
uPA	+	Array
Signal transduction		
Neuregulins	+	Array
Small GTPase Ran	+	2D
Rho GDI	+	2D
DJ-1, a positive regulator of AR signaling	+	2D
IGFBP-3	−	Array
Ser/Thr protein phosphatase 2A	−	2D
Transcription factors		
Fra-1	+	Array
c-Jun	+	Array
Metabolism		
L-Lactate dehydrogenase	−	2D
Isocitrate dehydrogenase	−	2D

[a] uPA, urokinase plasminogen activator; MMP-2, matrix metalloproteinase 2; IGFBP-3, insulin-like growth factor binding protein 3; AR, androgen receptor.
[b] +, up-regulation; −, down-regulation.
[c] 2D, 2D PAGE combined with mass spectrometry.

of vimentin and down-regulation of keratins indicate that Ec1WVM-mediated alterations in the gene expression pattern can be classified as EMT.

Effects of Ec1WVM on transcription of different genes require different duration of Ec1WVM expression. The observed effects on gene expression mediated by the E-cadherin mutant may either be direct or require prolonged inhibition of intercellular adhesion. To discriminate between these possibilities, we generated A431 clones with the DOX-regulated expression of Ec1WVM (clone 31D6; TET-on system). In cells treated with DOX, synthesis of Ec1WVM was induced as early as in 6 h, and alterations in cell morphology became evident at 24 h. At 48 h, Ec1WVM decreased the level of endogenous E-cadherin and induced full morphological transition of 31D6 cells, whose appearance became very similar to that of cells stably expressing Ec1WVM (Fig. 3A). 31D6 cells were maintained in the presence of DOX for different time periods or without DOX, and the expression of Ec1WVM-dependent genes was examined by Northern blot hybridization. Genes encoding components of intermediate filaments were not affected by Ec1WVM even after 16 days of induction (data not shown). Down-regulation of *igfbp-3* and *S100A4* genes occurred gradually, and after 16 days of incubation in the presence of DOX, expression of these genes reached levels comparable to those observed in stable clones (Fig. 3B). Conversely, *fra-1* and c-*jun* transcription was activated already after 48 h of stimulation by DOX, concomitant with morphological transformation of 31D6 cells.

Both genes immediately activated by Ec1WVM encode proteins, which belong to the AP-1 transcription factor family.

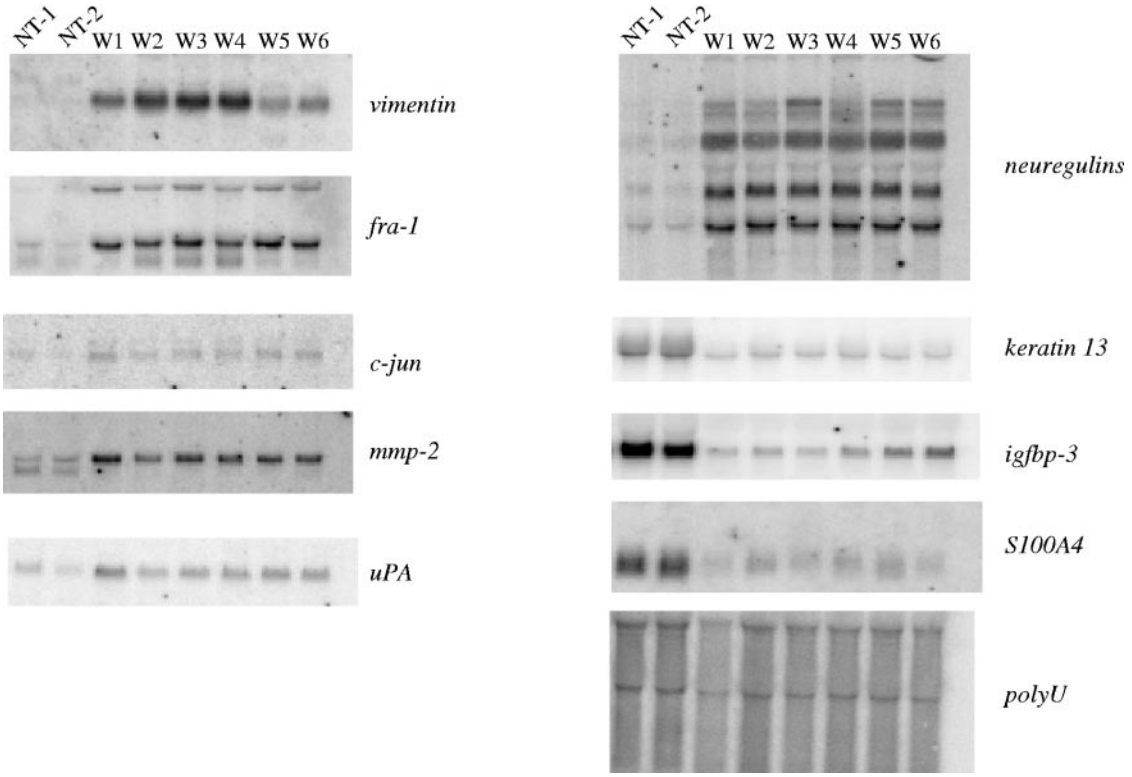

FIG. 2. Validation of Atlas cDNA microarray data. Transcription of genes identified in W1 to W6, NT-1, and NT-2 clones was analyzed by Northern blotting. Equal loading was verified by hybridization with the labeled *polyU* probe.

Consistent with their up-regulation by Ec1WVM in 31D6 cells, a reporter driven by a synthetic AP-1-dependent promoter (pTREx5Luc) was activated by DOX more than 4.5-fold. In contrast, DOX treatment did not influence transcriptional activity of a viral promoter located in the long terminal repeat of Rous sarcoma virus (Fig. 3C). Recently, Conacci-Sorrell et al. reported that functional inhibition of E-cadherin in SW480 cells resulted in nuclear translocation of β-catenin and activation of β-catenin-mediated transcription (15). As both *fra-1* and c-*jun* genes can be activated by β-catenin signaling (36), we examined whether this pathway was stimulated in 31D6 cells upon DOX treatment. However, a TOPFLASH/FOPFLASH reporter assay performed in DOX-treated or untreated 31D6 cells has demonstrated no effect of Ec1WVM on β-catenin/TCF-dependent transcription (Fig. 3D). Similarly, no activation of β-catenin signaling was observed in stable W2 and W3 clones (data not shown). Therefore, activation of AP-1 by Ec1WVM does not involve β-catenin signaling.

Prolonged and short-term (to a lesser extent) expression of Ec1WVM activates tumor cell migration. Prolonged and short-term expression of Ec1WVM in A431 cells resulted in loss of an epithelial pattern of cell growth and in cell dissociation (Fig. 1 and 3). Prolonged Ec1WVM expression down-regulated keratins and activated expression of vimentin (Fig. 2 and Table 1). Since cells undergoing EMT acquire a migratory phenotype, we hypothesized that Ec1WVM may affect cell motility and lead to increased cell migration into a wound. To test this, wounds were created in confluent cultures of NT-2, W2, and 31D6 cells and 31D6 cells pretreated with DOX for 48 h, and closure of wounds was monitored after 8 and 17 h. As expected, cells expressing Ec1WVM displayed accelerated wound closure compared with NT-2 and 31D6 cells maintained in the absence of DOX (Fig. 4). Whereas migration of clones with epithelial morphology closed wounds by approximately 50% in 17 h, wounds disappeared in W2 cell cultures. DOX-treated 31D6 cells exhibited an intermediate motility, and in 17 h they migrated approximately 1.7-fold faster than untreated cells. The moderate activation of cell motility in DOX-treated 31D6 cells was statistically significant. On the other hand, DOX produced no effect on migration of stable W3 and NT-2 clones (data not shown).

Transcriptional effects of Ec1WVM require AP-1 activity. Transcription of two members of the AP-1 transcription factor family, *fra-1* and c-*jun*, was activated by Ec1WVM as early as 48 h upon DOX stimulation of 31D6 cells. Positive autoregulatory loops are known to activate *fra-1* and c-*jun* transcription via AP-1-binding elements located in the *fra-1* intronic enhancer and c-*jun* gene promoter (1, 3). To test whether AP-1 activity is necessary for Ec1WVM-mediated up-regulation of *fra-1* and c-*jun* transcription, we employed a bicistronic DOX-sensitive vector pBI to generate a clone in which both Ec1WVM and the AP-1 dominant-negative mutant TAM67 fused with the enhanced green fluorescent protein (TAM67-GFP) are simultaneously induced by DOX (Fig. 5A, clone G10). Simultaneous induction of both Ec1WVM and TAM67 mutant proteins in G10 cells resulted in cell dissociation. There were no clear differences in cell morphology between DOX-treated G10 and 31D6 cells (compare Fig. 3A with 5A). Ex-

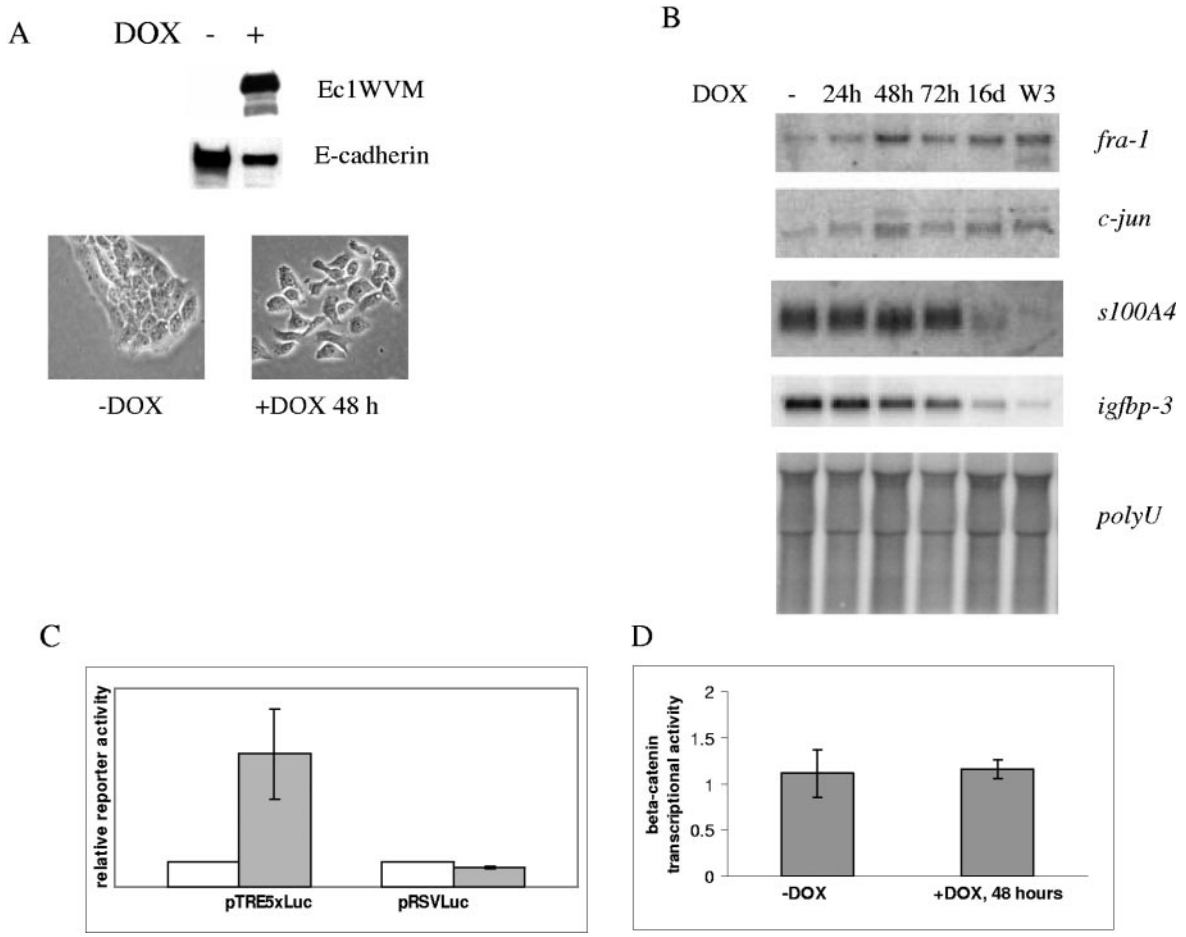

FIG. 3. Ec1WVM mutant induces rapid response in A431 cells. (A) Characterization of the 31D6 clone with DOX-regulated expression of Ec1WVM. Induction of Ec1WVM by DOX treatment for 48 h results in cell dissociation and morphological alterations. Immunoblot analysis of Ec1WVM and endogenous E-cadherin expression is shown in the upper part of the panel. 31D6 cells were maintained in the presence or absence of DOX for 48 h and analyzed with anti-myc and anti-E-cadherin antibodies. (B) Ec1WVM affects transcription of *fra-1*, c-*jun*, *S100A4*, and *igfbp3* in 31D6 cells. Total RNA was extracted from 31D6 cells maintained without DOX or with DOX for the indicated periods of time. Gene expression was examined by Northern blotting using ^{32}P-labeled probes as indicated. The membrane was probed with labeled *polyU* probe to demonstrate equal loading. (C) Ec1WVM activates AP-1-driven transcription in 31D6 cells. 31D6 cells were transfected with the AP-1-dependent reporter pTREx5Luc or with pRSVLuc along with the control β-galactosidase-expressing vector pCMVβ-gal and maintained in the presence (+) or absence (−) of DOX. At 48 h posttransfection, luciferase activity was measured and normalized to the β-galactosidase activity. The results (average and standard deviations) are expressed as the relative activation of luciferase in DOX-treated cells (gray bars) compared to that in untreated cells (white bars). (D) Ec1WVM does not influence TCF/LEF transcriptional activity. 31D6 cells were transfected with pTOPFLASH or pFOPFLASH reporters along with pCMVβ-gal and maintained in the absence or presence of DOX for 48 h. Relative TCF/LEF transcriptional activity was defined as ratio of pTOPFLASH/pFOPFLASH luciferase activities normalized to the β-galactosidase level detected in each transfection. The results (means ± standard deviations) of three independent experiments are shown.

pression of TAM67 not only suppressed AP-1 activation by Ec1WVM but also significantly inhibited the basal AP-1 activity detected in nonstimulated cells. In addition, TAM67 effectively blocked the stimulatory effect of Ec1WVM on *fra-1* and c-*jun* gene transcription (Fig. 5B), suggesting that AP-1 activity is necessary for transcriptional activation of both AP-1 family members induced by the dominant-negative mutant of E-cadherin. Similarly, in a striking difference with 31D6 cells expressing only Ec1WVM, *igfbp-3* and *S100A4* transcription was not affected or only insignificantly affected in G10 cells even after 16 days of culturing in the presence of DOX (compare Fig. 3B with 5B). Therefore, Ec1WVM affects expression of *S100A4* and *igfbp-3* also in AP-1-dependent manner.

Next, we aimed to examine whether stimulation of AP-1 is sufficient for the transcriptional up-regulation of *fra-1* and c-*jun* genes in A431 cells. Since an AP-1 family member, c-Fos, was most efficient in inducing EMT in murine epithelial cells (19, 21) and has been shown to directly regulate *fra-1* expression (3, 37), we chose to generate a clone of A431 cells with inducible DOX-dependent expression of c-Fos (clone B4). Even though c-Fos strongly activated AP-1-regulated reporter in B4 cells, epithelial cell morphology was not affected (Fig. 5A). Treatment of B4 cells with DOX for 48 h was sufficient to activate transcription of *fra-1* but not c-*jun* (Fig. 5B). Given that TAM67 effectively blocked c-*jun* activation (Fig. 5B), we concluded that Ec1WVM-mediated activation of AP-1 was necessary but not sufficient to up-regulate c-*jun*. These data suggested that although both genes were activated by

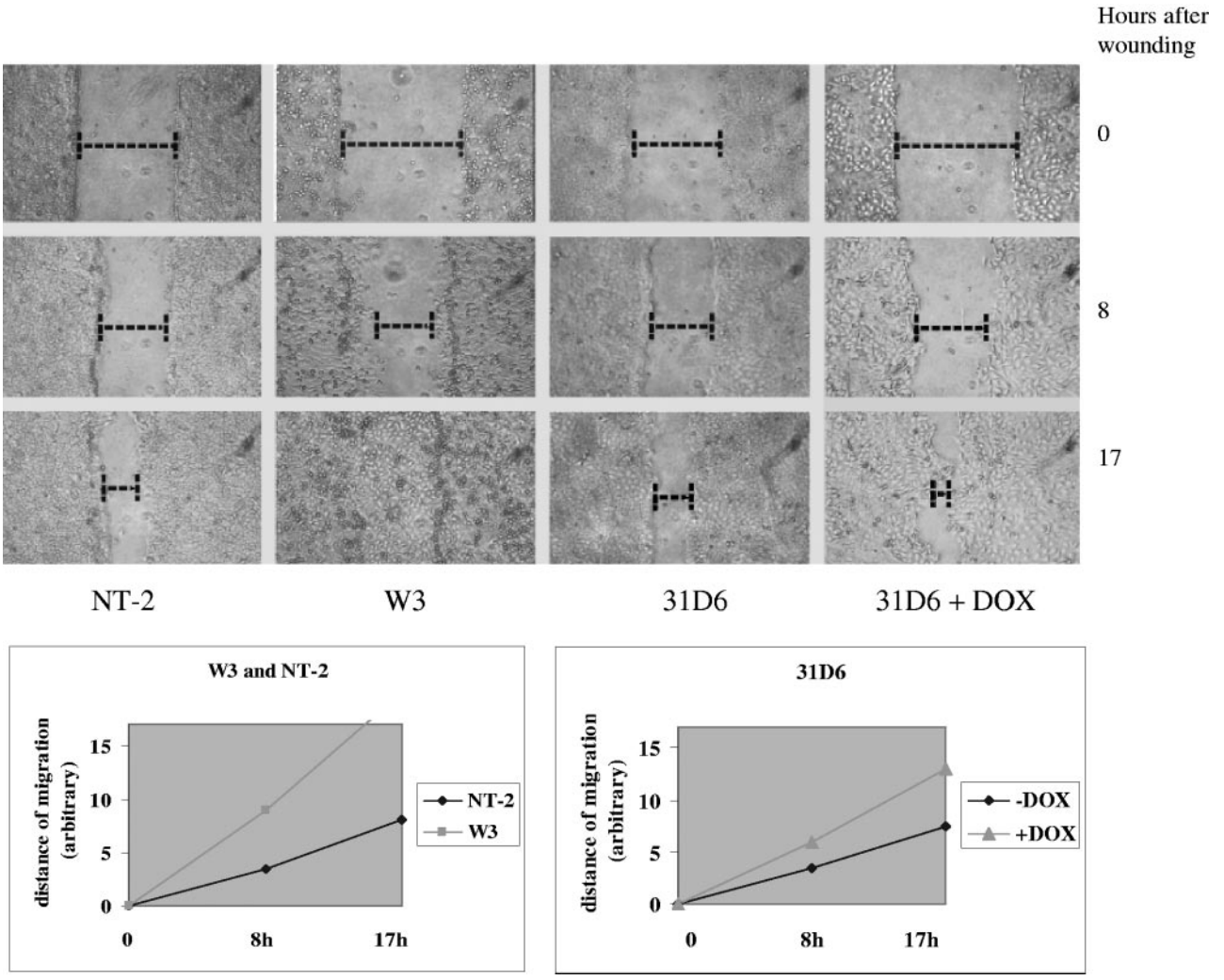

FIG. 4. Effect of Ec1WVM on tumor cell motility. Wounds were created in confluent cultures of NT-2, W3, and 31D6 cells cultured with (+) or without (−) DOX for 48 h prior the experiment. Wounds were marked and photographed after 0, 8, or 17 h. Experiments were repeated three times, and results of a typical experiment are shown. Wound closure at various time intervals was measured in arbitrary units and represented in graphs.

Ec1WVM in an AP-1-dependent manner, the exact mechanisms of transcriptional up-regulation of *fra-1* and c-*jun* were different.

AP-1 is critical for the activation of tumor cell motility by Ec1WVM. We aimed to evaluate whether the effect on tumor cell motility produced by Ec1WVM was AP-1 dependent. Migration into a wound of G10 or B4 cells either maintained without DOX or pretreated with DOX for 48 h was examined. As a positive control, we used a highly motile W3 cell line constitutively expressing Ec1WVM (Fig. 6). Expression of TAM67 not only counteracted the stimulatory effect of Ec1WVM on cell migration observed in 31D6 cells but also almost completely blocked cell motility (Fig. 6, clone G10). Moreover, activation of c-Fos in B4 cells was sufficient to stimulate cell migration into a wound (Fig. 6). Therefore, at early stages of EMT, Ec1WVM-mediated effects on tumor cell motility involve AP-1.

Next, we addressed the question whether the Ec1WVM-mediated activation of the two AP-1 family members Fra-1 and c-Jun contributes to the enhanced cell motility at later stages of EMT. W3 cells are very motile in wound-healing (Fig. 4 and 6) and transwell migration (data not shown) assays. They express a high level of vimentin and low levels of cytokeratins 13 and 15 and, therefore, can be considered as an end-point of Ec1WVM-induced EMT. We employed RNA interference to suppress the elevated expression of c-Jun and Fra-1 in W3 cells. By transfecting c-Jun and Fra-1-specific siRNAs, we inhibited expression of c-Jun and Fra-1 to levels similar to the level observed in parental A431 cells (Fig. 7A). The effects of single and double knockdowns on cell migration were evaluated in wound-healing and transwell migration assays. Transfection of W3 cells with the scrambled siRNA produced insignificant (if any) effect on cell migration in both wound-healing (compare Fig. 4 and 6 with 7B) and transwell migration assays (data not shown). However, in both assays, W3 cells with reduced Fra-1 and c-Jun expression levels migrated more slowly than cells transfected with the control siRNA (Fig. 7B and C). Although Fra-1 knockdown was more efficient than suppres-

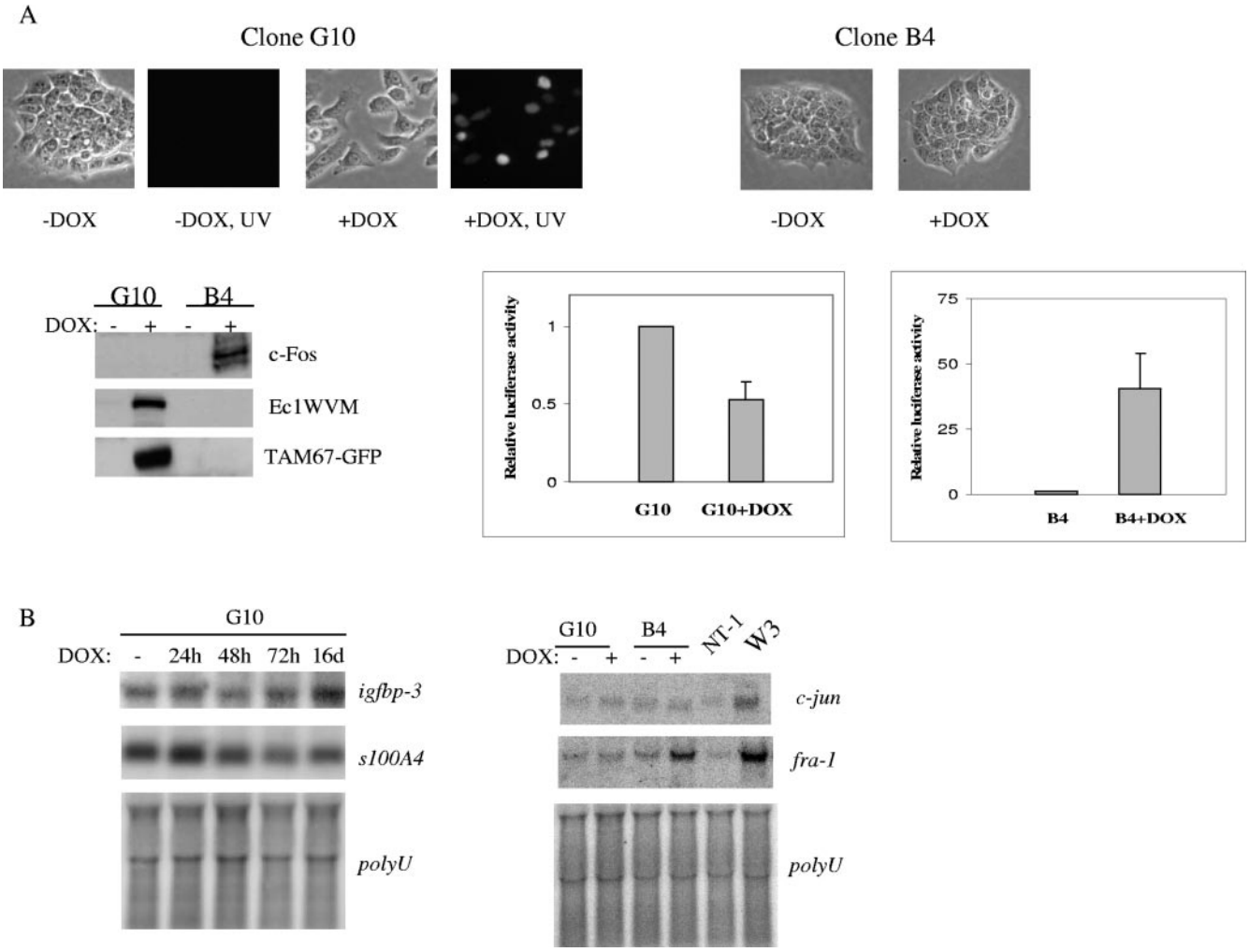

FIG. 5. AP-1 is involved in transcriptional effects of Ec1WVM. (A) Characterization of G10 and B4 clones. DOX induces morphological transformation of G10 but not B4 cells. Phase-contrast and fluorescence microscopy of cells cultured with or without DOX for 48 h is presented. Nuclear localization of TAM67-GFP in DOX-treated G10 cells is demonstrated. Expression of TAM67-GFP fusion protein, Ec1WVM, and c-Fos was examined in DOX-treated or untreated G10 and B4 cells by Western blotting with anti-c-Fos, anti-GFP, and anti-myc antibodies. The effect of DOX treatment on AP-1-dependent transcription in B4 and G10 cells is shown. Cells were transiently transfected with the AP-1-regulated reporter pTREx5Luc, and the activity was determined as described in the legend to Fig. 3. (B) Northern blot analysis of *S100A4*, *igfbp-3*, c-*jun*, and *fra-1* gene expression in G10 and B4 clones. G10 cells were cultured without DOX or with DOX treatment for indicated time periods (left). RNA was isolated, blotted, and hybridized to *S100A4* and *igfbp-3* probes. A Northern blot hybridization of RNA from G10 and B4 cells untreated or treated with DOX for 48 h is shown (right). RNA was hybridized to labeled probes as indicated. Hybridization to *polyU* confirms equal loading.

sion of c-Jun, both knockdowns produced similar effects on wound closure (Fig. 7B). In transwell assays, cells transfected with siRNA specific for c-Jun migrated even somewhat more slowly than cells with suppressed Fra-1 expression (Fig. 7C). Simultaneous knockdown of c-Jun and Fra-1 resulted in the most efficient inhibition of cell motility (Fig. 7B and C). Therefore, enhanced expression of Fra-1 and c-Jun was critically important to maintain enhanced motility of W3 cells.

Ec1WVM does not increase the level of phosphorylated EGFR in A431 cells. Recent work by Qian et al. has demonstrated that the ligand-dependent activation of EGFR and another RTK is negatively regulated by E-cadherin in an adhesion-dependent manner (48). As in a variety of cell types, activation of RTK ultimately results in activation of AP-1, and given that A431 cells express EGFR at very high levels, we hypothesized that Ec1WVM activates AP-1 via EGFR. To test this hypothesis, we evaluated expression levels of phosphorylated EGFR in stable W2 and NT-2 clones and in 31D6 cells maintained in the absence or in the presence of DOX for 48 h. However, these experiments clearly demonstrated that Ec1WVM does not increase the level of phospho-EGFR in A431 cells (Fig. 8). Moreover, treatment of cells with EGF at different concentrations resulted in activation of EGFR independently of Ec1WVM expression. Thus, activation of AP-1 by Ec1WVM was EGFR independent.

DISCUSSION

EMT underlies dispersing cell lineages in embryonic development and contributes to progression of carcinoma during epithelial tumorigenesis (52, 59, 60). Although a number of in

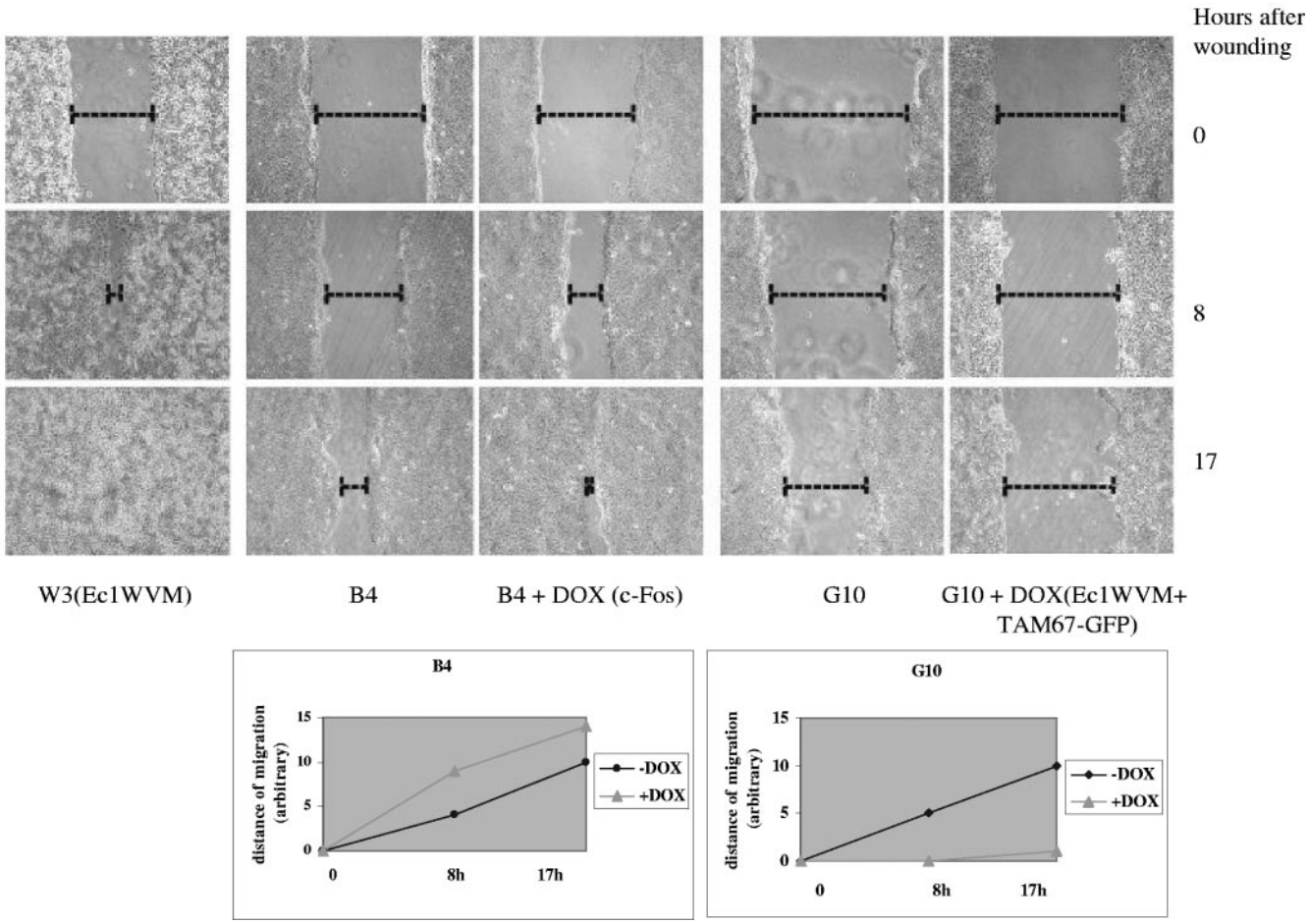

FIG. 6. AP-1 controls motility of B4 and G10 cells. G10 and B4 cells were cultured with or without DOX. Where indicated, DOX was added 48 h prior to the experiment. Wounds were made in confluent cell cultures, marked, and photographed after indicated time periods. Wound closure at various time intervals was measured in arbitrary units and represented in graphs. Ectopic expression of c-Fos, Ec1WVM, and TAM67-GFP is indicated in brackets.

vitro models of EMT exist, there is no clear consensus on the definition of this phenomenon. In most studies, loss of epithelial polarity accompanied by an increase in cell motility, repression of the epithelial markers E-cadherin and cytokeratins, and activation of the mesenchymal marker vimentin is considered as EMT. In in vitro models of epithelial cancer, EMT can be initiated by various groups of signaling molecules. These include growth factors (EGF, hepatocyte growth factor, transforming growth factor β, or fibroblast growth factor 2) (34, 57, 59), transcription factors (c-Fos, c-Jun, Snail, Slug, ZEB-1, ZEB-2/SIP1, and E47) (2, 6, 8, 14, 19, 20, 21), small GTPases (Ras and Rac) (18, 33), or protein kinases, such as constitutively activated MEK (53). Here, we have demonstrated that prolonged inhibition of E-cadherin function is sufficient for induction of morphological conversion and stimulation of tumor cell motility. By two approaches, we found that stable expression of a dominant-negative E-cadherin mutant, Ec1WVM, alters gene expression pattern. Given that down-regulation of keratins 15 and 13 and activation of vimentin has been observed, we concluded that Ec1WVM induced a complete EMT in A431 cells. Our data show that several phases can be delineated in Ec1WVM-mediated EMT. Morphological alterations, a moderate but statistically significant increase in cell motility, and activation of AP-1 occurred within 24 to 48 h. Immunoprecipitation experiments demonstrated that these effects were concomitant with the replacement of wild-type E-cadherin by a mutant in adhesive complexes (data not shown). The extended (3 to 16 days) expression of Ec1WVM resulted in down-regulation of *S100A4* and *igfbp-3*, which required AP-1 activity. The majority of changes in gene expression (including up-regulation of vimentin and repression of cytokeratins) were observed only in stable clones but not in 31D6 cells even after 16 days of cultivation in the presence of DOX. In addition, cells constitutively expressing Ec1WVM more rapidly migrated into wounds than DOX-treated 31D6 cells. We therefore conclude that completion of EMT requires prolonged (more than 16 days) inhibition of E-cadherin function.

Transcriptional up-regulation of *fra-1* and c-*jun* and functional activation of AP-1 are early events in Ec1WVM-mediated EMT. Stimulation of *fra-1* and c-*jun* transcription can be blocked by TAM67-GFP (Fig. 5B), suggesting that Ec1WVM activates a positive autoregulatory mechanism that keeps AP-1 activity elevated in cells with compromised cell-cell adhesion.

A431 cells, as other cell lines derived from epithelial cancers,

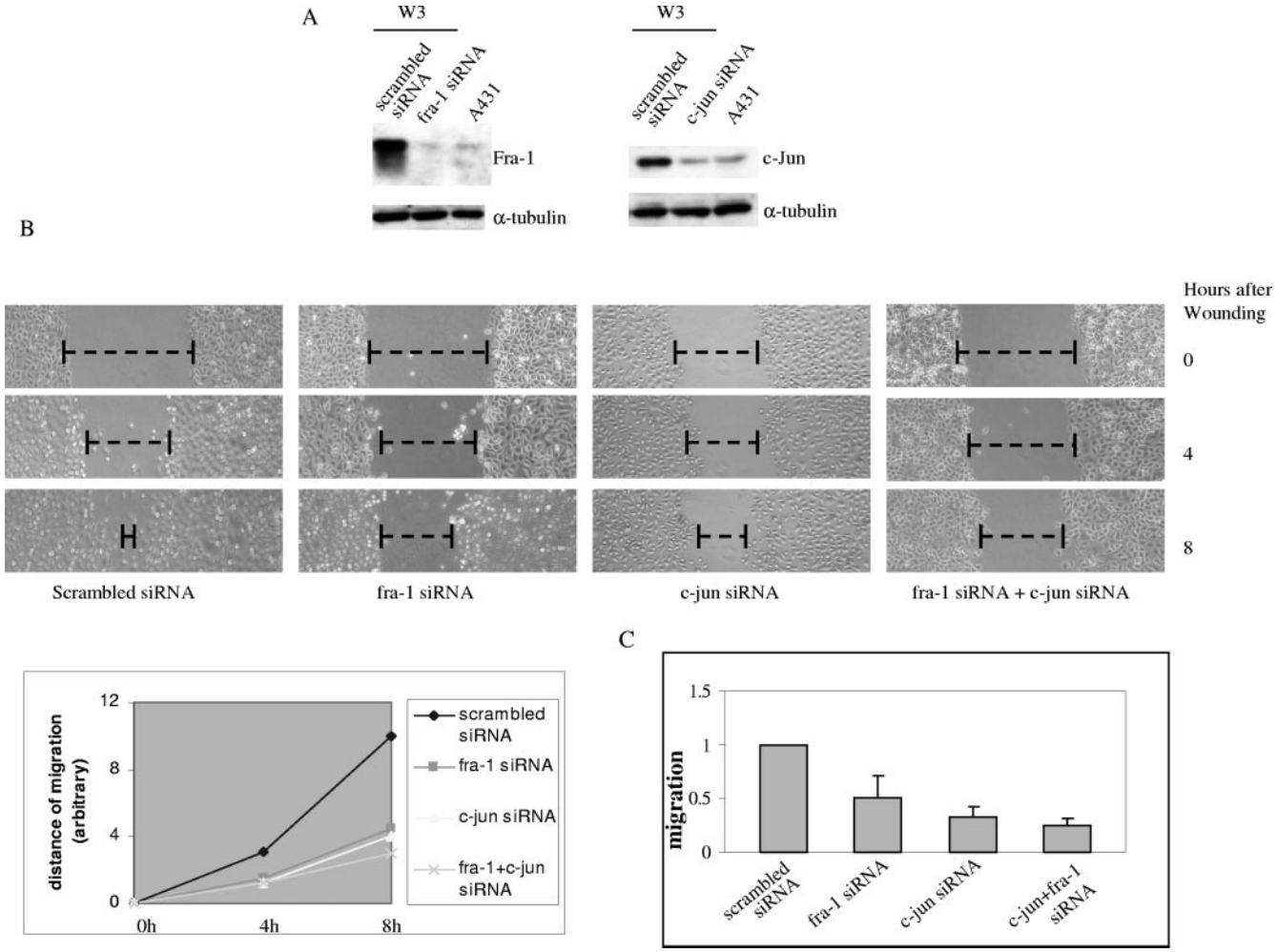

FIG. 7. AP-1 family members c-Jun and Fra-1 are essential for increased motility of W3 cells. (A) RNA interference-mediated inhibition of c-Jun and Fra-1 expression in W3 cells. Cells were transfected with siRNAs targeting c-Jun and Fra-1. Scrambled siRNA was used as a control. The extent of silencing was determined by Western blotting as indicated. (B) Knockdown of c-Jun or Fra-1 retards wound closure. W3 cells were transfected with scrambled siRNA or specific siRNA inhibiting c-Jun or Fra-1 expression. Cell migration was analyzed in wound-healing assays after indicated time intervals. (C) Cell migration was analyzed in transwell motility assay. Expression of c-Jun, Fra-1, or c-Jun and Fra-1 in combination was silenced by RNAi in W3 cells. A total of 10^5 cells were seeded onto 8 μM polycarbonate transwell filters and allowed to migrate toward fetal calf serum gradient. Cells that migrated to the lower surface of the filter were stained and counted microscopically. Migration was normalized to that of W3 cells transfected with the control siRNA. Data are means ± the standard deviations of triplicate experiments. The experiments were repeated three times with similar results.

migrate as cell aggregates, sheets, or clusters (collective migration). In this form of migration, aggregated cells move as a functional unit, in which subsets of active cells utilize actin-mediated ruffles and generate integrin-dependent traction. Other cells included in an aggregate are passively dragged forward by means of intercellular adhesion (reviewed in reference 22). Given that induction of c-Fos in clone B4 does not affect epithelial morphology but is sufficient to accelerate cell

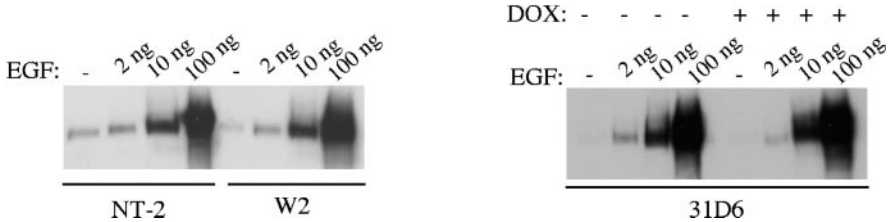

FIG. 8. Expression of Ec1WVM does not alter phosphorylation of EGFR. NT-2, W3, or 31D6 cells were serum depleted for 24 h and treated with indicated concentrations of EGF for 5 min. Expression of Ec1WVM in 31D6 cells was induced by adding DOX for 48 h. EGFR phosphorylation was detected in Western blotting using a phospho-specific antibody.

motility, we conclude that collective migration of epithelial cells is positively regulated by AP-1. This conclusion is consistent with data generated by Malliri et al. showing that prolonged expression of a dominant-negative mutant of c-Jun blocks motility of nonstimulated A431 cells (35). Loss of cell-cell adhesion during EMT results in a switch from collective toward different forms of more efficient individual migration patterns (22). TAM67-GFP effectively blocks cell motility activated by Ec1WVM at an early EMT phase (clone G10). Completion of EMT further contributes to enhanced cell motility (stable clones expressing Ec1WVM are more active in the wounding-healing assay than 31D6 cells pretreated with DOX for 48 h). By RNA interference we demonstrated that enhanced expression of c-Jun and Fra-1 is required for active migration of W3 cells, e.g., at later EMT stages. Taken together, these data clearly demonstrate that the role of AP-1 in cell motility is not restricted to the control of the epithelial type of cell migration. A positive autoregulatory loop, which is triggered by Ec1WVM and activates transcription of *fra-1* and c-*jun* genes, is essential for enhanced cell motility at different stages of EMT.

We were interested to identify Ec1WVM-mediated signaling providing an initial activating stimulus to the preexisting AP-1 complexes. Since abundance, activity, and composition of AP-1 complex is controlled by MAPK, we examined whether expression and phosphorylation levels of MAPK are affected by DOX in 31D6 cells. Even though we did observe a moderate increase in the phosphorylation level of MAPKs in DOX-treated 31D6 cells (data not shown), the exact molecular events triggering induction of AP-1 by Ec1WVM remain unclear. A431 cells express high levels of EGFR and are capable of autocrine stimulation of this receptor. As E-cadherin-mediated adhesion may inhibit ligand-dependent activation of RTK (48), we hypothesized that the application of Ec1WVM would result in activation of EGFR in the A431 cell system. However, Ec1WVM had no effect on phosphorylation of EGFR in DOX-stimulated 31D6 cells (Fig. 8), suggesting that RTK pathways are unlikely to be involved in the activation of AP-1 by Ec1WVM. Nor is β-catenin signaling, known to activate *fra-1* and c-*jun* gene transcription, involved in Ec1WVM-mediated activation of AP-1 (Fig. 3D). One of the hallmarks of EMT is the reorganization of the actin-based cytoskeleton, which reflects loss of epithelial polarity and a switch from cell-cell to cell-substratum interactions. Recently, we found that expression of Ec1WVM in c-Fos-transformed murine epithelioid carcinoma cells resulted in increased cell adhesion to the extracellular matrix components (J. Mejlvang et al., unpublished data). Therefore, we suggest that Ec1WVM may affect cell-substratum interactions also in the A431 cell system, stimulating integrin signaling and hence triggering the initial AP-1 activation. The documented reciprocity between the level of organization of adherens junctions and focal adhesions (31), as well as previously described cross talks between E-cadherin and specific integrin receptors (65), supports this hypothesis.

EMT-inducing transcription factors Snail, Slug, ZEB-2/SIP1, or E47 directly inhibit the E-cadherin gene promoter. Emerging evidence suggests that these transcriptional repressors act downstream of a variety of EMT-initiating signals to down-regulate E-cadherin gene transcription (15, 20, 23, 45). In addition to transcriptional repression, several other genetic and epigenetic mechanisms may be responsible for inactivation of E-cadherin-dependent cell-cell adhesion in human cancers. E-cadherin function can be inhibited by gene mutations, promoter polymorphisms, promoter hypermethylation, and loss of the E-cadherin locus (4, 17, 26, 66). For instance, in poorly differentiated diffuse-type gastric cancer and lobular breast carcinoma, mutations affecting the extracellular E-cadherin domain have been observed. Our data suggest that structural mutations in the E-cadherin gene or consistent cleavage of E-cadherin extracellular domains chronically exposed to matrix metalloproteinases secreted by stromal cells (32) may be sufficient to trigger a process ultimately leading to EMT in tumor cells. Often, cells respond relatively rapidly to EMT-initiating signals. For example, 5 days of chronic EGF treatment is sufficient to induce morphological transformation and to down-regulate epithelial markers in A431 cells (34). In the same cell line, the transcription factor ZEB-2/SIP1 induced full EMT as rapidly as within 48 h (our unpublished data). In contrast, EMT induced by the dominant-negative E-cadherin mutant is a slow process. Different kinetics of EMTs mediated by an E-cadherin mutant and its transcriptional repressors may indicate that the repressors directly inhibit transcription of other epithelial genes and, therefore, have broader functions in EMT. In support of this, Snail has been shown to down-regulate tight junction components independently of E-cadherin down-regulation (41). Interestingly, rapid EMT of MDCK cells mediated by ectopic expression of Snail involves inhibition of G_1/S cell cycle progression (62). A similar effect of exogenous ZEB-2/SIP1 on retinoblastoma protein-dependent cell cycle regulation was observed in the A431/SIP1 model (our unpublished data). This suggests that cells retaining control over G_1/S transition and undergoing a rapid EMT acquire a growth disadvantage. In contrast, neither the cell proliferation rate nor cell cycle progression was affected in the EMT model reported here (data not shown). Therefore, it is plausible to speculate that SIP1 or Snail induces either transient EMT or stable EMT only in those cells in which control over G_1/S transition has been lost. Gradual EMT initiated by mutations of the components of E-cadherin complex or by cleavage of E-cadherin by proteases may be a prevalent mechanism of stable EMT in cancer cells, in which the control over G_1/S transition is not completely compromised (such as A431 or MDCK cells).

Prolonged inhibition of epithelial adhesion alters expression of several genes that are critical players in signal transduction pathways controlling tumor cell motility and invasive growth. The challenge is to further elucidate molecular mechanisms linking inhibition of epithelial cell adhesion with the alterations in cell signaling networks. This may lead to the design of novel methods uncoupling the loss of E-cadherin from tumor cell invasion and metastasis.

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REFERENCES

1. **Angel, P., K. Hattori, T. Smeal, and M. Karin.** 1988. The c-jun proto-oncogene is positively autoregulated by its product, jun/AP-1. Cell **55:**875–885.
2. **Batlle, E., E. Sancho, C. Franci, D. Dominguez, M. Monfar, J. Baulida, A. De Herreros.** 2000. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat. Cell. Biol. **2:**84–89.
3. **Bergers, G., P. Graninger, S. Braselmann, C. Wrighton, and M. Busslinger.** 1995. Transcriptional activation of the *fra-1* gene by AP-1 is mediated by regulatory sequences in the first intron. Mol. Cell. Biol. **15:**3748–3758.
4. **Berx, G., K. Becker, H. Hofler, and F. van Roy.** 1998. Mutations of the human E-cadherin (CDH1) gene. Hum. Mutat. **12:**226–237.
5. **Betson, M., E. Lozano, J. Zhang, and V. Braga.** 2002. Rac activation upon cell-cell contact formation is dependent on signaling from the epidermal growth factor receptor. J. Biol. Chem. **277:**36962–36969.
6. **Bolos, V., H. Peinado, M. Perez-Moreno, M. Fraga, M. Esteller, and A. Cano.** 2003. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J. Cell Sci. **116:**499–511.
7. **Caca, K., F. Kolligs, J. X. M. Hayes, J. Qian, A. Yahanda, D. Rimm, J. Costa, and E. Fearon.** 1999. Beta- and gamma-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. Cell. Growth Differ. **10:**369–376.
8. **Cano, A., M. A. Pérez-Moreno, I. Rodrigo, A. Locascio, M. J. Blanco, M. G. del Barrio, F. Portillo, and M. A. Nieto.** 2000. The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat. Cell. Biol. **2:**76–83.
9. **Cavallaro, U., and G. Christofori.** 2004. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat. Rev. Cancer. **4:**118–132.
10. **Celis, J. E., P. Gromov, T. Cabezon, J. Moreira, N. Ambartsumian, K. Sandelin, F. Rank, I. Gromova.** 2004. Proteomic characterization of the interstitial fluid perfusing the breast tumor microenvironment: a novel resource for biomarker and therapeutic target discovery. Mol. Cell Proteomics **3:**327–344.
11. **Celis, J. E., S. Trentemølle, and P. Gromov.** Gel-based proteomics: high resolution two-dimensional gel electrophoresis of proteins. Isoelectric focusing (IEF) and nonequilibrium pH gradient electrophoresis (NEPHGE). *In* J. E. Celis, N. Carter, T. Hunter, D. Shotton, K. Simons and J. V. Small (ed.), Cell biology, a laboratory handbook, vol. 4, 3rd ed., in press. Academic Press, San Diego, Calif.
12. **Chitaev, N., S. Troyanovsky.** 1998. Adhesive but not lateral E-cadherin complexes require calcium and catenins for their formation. J. Cell Biol. **142:**837–846.
13. **Cohn, M., I. Hjelmso, L.-C. Wu, P. Guldberg, E. Lukanidin, and E. Tulchinsky.** 2001. Characterization of Sp1, AP-1, CBF and KRC binding sites and minisatellite DNA as functional elements of the metastasis-associated mts1/S100A4 gene intronic enhancer. Nucleic Acids Res. **29:**3335–3346.
14. **Comijn, J., G. Berx, P. Vermassen, K. Verschueren, L. van Grunsven, E. Bruyneel, M. Mareel, D. Huylebroeck, and F. van Roy.** 2001. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol. Cell **7:**1267–1278.
15. **Conacci-Sorrell, M., I. Simcha, T. Ben-Yedida, J. Blechman, P. Savanger, and A. Ben-Ze'ev.** 2003. Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of b-catenin signalling, Slug and MAPK. J. Cell Biol. **163:**847–857.
16. **Conacci-Sorrell, M., J. Zhurinsky, and A. Ben-Ze'ev.** 2002. The cadherin-catenin adhesion system in signalling and cancer. J. Clin. Investig. **109:**987–991.
17. **Di Croce, L., and P. Pelicci.** 2003. Tumour-associated hypermethylation: silencing E-cadherin expression enhances invasion and metastasis. Eur. J. Cancer. **39:**413–414.
18. **Edme, N., J. Downward, J.-P. Thiery, and B. Boyer.** 2002. Ras induces NBT-II epithelial cell scattering through the coordinate activities of Rac and MAPK pathways. J. Cell Sci. **115:**2591–2601.
19. **Eger, A., A. Stockinger, B. Schaffhauser, H. Beug, and R. Foisner.** 2000. Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity. J. Cell Biol. **148:**173–188.
20. **Eger, A., K. Aigner, S. Sonderegger, B. Dampier, S. Oehler, M. Schreiber, G. Berx, A. Cano, H. Beug, and R. Foisner.** 2005. DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. Oncogene **24:**2375–2385.
21. **Fialka, I., H. Schwarz, E. Reichmann, M. Oft, M. Busslinger, H. Beug.** 1996. The estrogen-dependent c-JunER protein causes a reversible loss of mammary epithelial cell polarity involving a destabilization of adherens junctions. J. Cell Biol. **132:**1115–1132.
22. **Friedl, P., and K. Wolf.** 2003. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev. Cancer **3:**362–374.
23. **Fujita, N., D. Jaye, M. Kajita, C. Geigerman, C. Moreno, and P. Wade.** 2003. MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell **113:**207–219.
24. **Gottardi, C., E. Wong, and B. Gumbiner.** 2001. E-cadherin suppresses cellular transformation by inhibiting β-catenin signalling in an adhesion-independent manner. J. Cell Biol. **153:**1049–1060.
25. **Gromova, I., and. J. E. Celis.** Protein detection in gels by silver staining: a procedure compatible with mass-spectrometry in cell biology. *In* J. E. Celis, N. Carter, T. Hunter, D. Shotton, K. Simons and J. V. Small (ed.), Cell biology, a laboratory handbook, vol. 4, 3rd ed., in press. Academic Press, San Diego, Calif.
26. **Guilford, P., J. Hopkins, J. Harraway, M. McLeod, N. McLeod, P. Harawira, H. Taite, R. Scoular, A. Miller, and A. Reeve.** 1998. E-cadherin germline mutations in familial gastric cancer. Nature **392:**402–405.
27. **Hennigan, R., and P. Stambrook.** 2001. Dominant negative c-jun inhibits activation of the cyclin D1 and cyclin E kinase complexes. Mol. Biol. Cell **12:**2352–2363.
28. **Hoschuetzky, H., H. Aberle, and R. Kemler.** 1994. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. J. Cell Biol. **127:**1375–1380.
29. **Huelsken, J., and W. Birchmeier.** 2001. New aspects of Wnt signaling pathways in higher vertebrates. Curr. Opin. Genet. Dev. **11:**547–553.
30. **Kovacs, E. M., R. Ali, A. McCormack, and A. Yap.** 2002. E-cadherin homophilic ligation directly signals through Rac and phosphatidylinositol 3-kinase to regulate adhesive contacts. J. Biol. Chem. **277:**6708–6718.
31. **Levenberg, S., B.-Z. Katz, K. Yamada, and B. Geiger.** 1998. Long-range and selective autoregulation of cell-cell or cell-matrix adhesions by cadherin or integrin ligands. J. Cell Sci. **111:**347–357.
32. **Lochter, A., S. Galosy, J. Muschler, N. Freedman, Z. Werb, and M. Bissel.** 1997. Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and premalignant phenotype in mammary epithelial cells. J. Cell Biol. **139:**1861–1872.
33. **Lozano, E., M. Betson, and V. Braga.** 2003. Tumor progression: small GTPases and loss of cell-cell adhesion. Bioessays **25:**452–463.
34. **Lu, Z., S. Ghosh, Z. Wang, and T. Hunter.** 2003. Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. Cancer Cell **4:**499–515.
35. **Malliri, A., M. Symons, R. Hennigan, A. Hurlstone, R. Lamb, T. Wheeler, and B. Ozanne.** 1998. The transcription factor AP-1 is required for EGF-induced activation of Rho-like GTPases, cytoskeletal rearrangements, motility, and in vitro invasion of A431 cells. J. Cell Biol. **143:**1087–1099.
36. **Mann, B., M. Gelos, A. Siedow, M. Hanski, A. Gratchev, M. Ilyas, W. Bodmer, M. Moyer, E. Riecken, H. Buhr, and C. Hanski.** 1999. Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc. Natl. Acad. Sci. USA **96:**1603–1608.
37. **Matsuo, K., J. Ownes, M. Tonko, C. Elliott, T. Chambers, and E. Wagner.** 2000. Fosl1 is a transcriptional target of c-Fos during osteoclast differentiation. Nat. Genet. **24:**184–187.
38. **Nieman, M., J. B. Kim, K. R. Johnson, and M. J. Wheelock.** 1999. Mechanism of extracellular domain-deleted dominant negative cadherins. J. Cell Sci. **112:**1621–1632.
39. **Noren, N., W. Arthur, and K. Burridge.** 2003. Cadherin engagement inhibits RhoA via p190RhoGAP. J. Biol. Chem. **278:**13615–13618.
40. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
41. **Ohkubo, T., and M. Ozawa.** 2004. The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation. J. Cell Sci. **117:**1675–1685.
42. **Orsulic, S., O. Huber, H. Aberle, S. Arnold, and R. Kemler.** 1999. E-cadherin binding prevents β-catenin nuclear localisation and β-catenin/LEF-1-mediated transactivation. J. Cell Sci. **112:**1237–1245.
43. **Pece, S., M. Chiariello, C. Murga, and J. Gutkind.** 1999. Activation of the protein kinase Akt/PKB by the formation of E-cadherin-mediated cell-cell junctions. Evidence for the association of phosphatidylinositol 3-kinase with the E-cadherin adhesion complex. J. Biol. Chem. **274:**19347–19351.
44. **Pece, S., and J. Gutkind.** 2000. Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. J. Biol. Chem. **275:**41227–41233.
45. **Peinado, H., M. Quintanilla, and A. Cano.** 2003. Transforming growth factor β-1 induces snail transcription factor in epithelial cell lines. J. Biol. Chem. **278:**21113–21123.
46. **Perez-Moreno, M. A., A. Locascio, I. Rodrigo, G. Dhondt, F. Portillo, M. A. Nieto, and A. Cano.** 2001. A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. J. Biol. Chem. **27629:**27424–27431.
47. **Polakis, P.** 2000. Wnt signalling and cancer. Genes Dev. **14:**1837–1851.
48. **Qian, X., T. Karpova, A. Sheppard, J. McNally, and D. Lowy.** 2004. E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. EMBO J. **21:**1739–1784.
49. **Rappsilber, J., Y. Ishihama, and M. Mann.** 2003. Stop and go extraction tips

for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. **75:**663–670.
50. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
51. **Sanson, B., P. White, and J.-P. Vincent.** 1996. Uncoupling cadherin-based adhesion from wingless signalling in *Drosophila*. Nature **383:**627–630.
52. **Savagner, P.** 2001. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays **23:**912–923.
53. **Schramek, H., E. Feifel, E. Healy, and V. Pollack.** 1997. Constitutively active mutant of the mitogen-activated protein kinase kinase MEK1 induces epithelial dedifferentiation and growth inhibition in Madin-Darby canine kidney-C7 cells. J. Biol. Chem. **272:**11426–11433.
54. **Shapiro, L., A. M. Fannon, P. D. Kwong, A. Thompson, M. S. Lehmann, G. Grubel, J.-F. Legrand, J. Als-Neilsen, D. R. Colman, and W. A. Hendrickson.** 1995. Structural basis of cell-cell adhesion by cadherins. Nature **374:**327–337.
55. **Shevchenko, A., M. Wilm, O. Vorm, and M. Mann.** 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. **68:**850–858.
56. **St. Croix, B., C. Sheehan, J. Rak, V. Florenes, J. Slingerland, and R. Kerbel.** 1998. E-cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). J. Cell Biol. **142:**557–571.
57. **Stockinger, A., A. Eger, J. Wolf, H. Beug, and R. Foisner.** 2001. E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity. J. Cell Biol. **154:**1185–1196.
58. **Takeichi, M.** 1993. Cadherins in cancer: implications for invasion and metastasis. Curr. Opin. Cell Biol. **5:**806–811.
59. **Thiery, J.-P.** 2003. Epithelial-mesenchymal transitions in development and pathologies. Curr. Opin. Cell Biol. **15:**740–746.
60. **Thiery, J.-P., and D. Chopin.** 1999. Epithelial cell plasticity in development and tumor progression. Cancer Metastasis Rev. **18:**31–42.
61. **van de Wetering, M., N. Barker, I. Harkes, M. van der Heyden, N. Dijk, A. Hollestelle, J. Klijn, H. Clevers, and M. Schutte.** 2001. Mutant E-cadherin breast cancer cells do not display constitutive Wnt signaling. Cancer Res. **61:**278–284.
62. **Vega, S., A. Morales, O. Ocaña, F. Valdés, I. Fabregat, and M. A. Nieto.** 2004. Snail blocks the cell cycle and confers resistance to cell death. Genes Dev. **118:**1131–1143.
63. **Vial, E., E. Sahai, and C. J. Marshall.** 2003. ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility. Cancer Cell **4:**67–79.
64. **Vleminckx, K., and R. Kemler.** 1999. Cadherins and tissue formation: integrating adhesion and signalling. Bioessays **21:**211–220.
65. **von Schlippe, M., J. Marshall, P. Perry, M. Stone, A. J. Zhu, and I. R. Hart.** 2000. Functional interaction between E-cadherin and av-containing integrins in carcinoma cells. J. Cell Sci. **113:**425–437.
66. **Wang, H.-D., J. Ren, and L. Zhang.** 2004. CDH1 germline mutation in hereditary gastric carcinoma. World J. Gastroenterol. **10:**3088–3093.
67. **Wong, A., and B. Gumbiner.** 2003. Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. J. Cell Biol. **161:**1191–1203.
68. **Zantek, N., M. Azimi, M. Fedor-Chaiken, B. Wang, R. Brackenbury, and M. Kinch.** 1999. E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. Cell Growth Differ. **10:**629–638.