

Expression of N-Cadherin by Human Squamous Carcinoma Cells Induces a Scattered Fibroblastic Phenotype with Disrupted Cell–Cell Adhesion

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Abstract. E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell–cell adhesion and plays an important role in maintaining the normal phenotype of epithelial cells. Disruption of E-cadherin activity in epithelial cells correlates with formation of metastatic tumors. Decreased adhesive function may be implemented in a number of ways including: (a) decreased expression of E-cadherin; (b) mutations in the gene encoding E-cadherin; or (c) mutations in the genes that encode the catenins, proteins that link the cadherins to the cytoskeleton and are essential for cadherin mediated cell–cell adhesion. In this study, we explored the possibility that inappropriate expression of a nonepithelial cadherin by an epithelial cell might also result in disruption of cell–cell adhesion. We showed that a squamous cell carcinoma–derived cell line expressed N-cadherin and displayed a scattered

fibroblastic phenotype along with decreased expression of E- and P-cadherin. Transfection of this cell line with antisense N-cadherin resulted in reversion to a normal-appearing squamous epithelial cell with increased E- and P-cadherin expression. In addition, transfection of a normal-appearing squamous epithelial cell line with N-cadherin resulted in downregulation of both E- and P-cadherin and a scattered fibroblastic phenotype. In all cases, the levels of expression of N-cadherin and E-cadherin were inversely related to one another. In addition, we showed that some squamous cell carcinomas expressed N-cadherin in situ and those tumors expressing N-cadherin were invasive. These studies led us to propose a novel mechanism for tumorigenesis in squamous epithelial cells; i.e., inadvertent expression of a nonepithelial cadherin.

THE cadherins are members of a large family of transmembrane glycoproteins that mediate calcium-dependent, homotypic cell–cell adhesion and play an important role in the maintenance of normal tissue architecture (reviewed in Takeichi, 1990). As the transmembrane component of the adherens junction, they are composed of three segments: (a) an extracellular domain responsible for cadherin–cadherin interaction; (b) a single-pass transmembrane domain; and (c) a highly conserved cytoplasmic domain that associates with actin filaments and thus serves to connect the outside of the cell to the cytoskeleton. Cadherins are not bound directly to the actin cytoskeleton, but rather, are connected indirectly via a group of proteins known as the catenins.

The catenins were identified as proteins coimmunoprecipitating with the classic cadherins, and were named α -catenin, β -catenin, and γ -catenin according to their mobility on SDS-PAGE (Nagafuchi and Takeichi, 1988; Ozawa

et al., 1989; Wheelock and Knudsen, 1991; McCrea et al., 1991; McCrea and Gumbiner, 1991). α -Catenin is a 102-kD protein that is associated with the cadherin indirectly through its interaction with β -catenin or γ -catenin. β -catenin is a 95-kD protein that shares ~65 percent identity with γ -catenin (Fouquet et al., 1992), an 82-kD protein also named plakoglobin (Knudsen and Wheelock, 1992; Peifer et al., 1992). β -Catenin and plakoglobin associate directly with the cadherin and can substitute for one another in the cadherin–catenin complex (Butz and Kemler, 1994; Hinck et al., 1994; Näthke et al., 1994; Sacco et al., 1995). Thus, the cell–cell adherens junction is a structure composed of the transmembrane cadherin, which is associated directly with either β -catenin or plakoglobin, which in turn associates directly with α -catenin. α -Catenin then mediates the interaction between the cadherin–catenin complex and the actin cytoskeleton (Nagafuchi et al., 1994; Knudsen et al., 1995; Rimm et al., 1995).

Numerous studies have demonstrated the importance of the E-cadherin/catenin complex in maintaining the normal phenotype of epithelial cells. Early studies showed that inhibiting E-cadherin activity with function-perturbing antibodies altered the morphology of MDCK cells and conferred upon them the ability to invade collagen gels and embryonic chicken heart tissue (Behrens et al., 1989; Chen

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and Öbrink, 1991). In addition, invasive fibroblast-like carcinoma cells could be converted to a noninvasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991).

Recently, several studies have reported a correlation between decreased function of the E-cadherin/catenin complex and the formation of human tumors. In some cases, decreased adhesive function was caused by decreased levels of expression of E-cadherin, and in other cases, by a mutation in the gene encoding E-cadherin such that the expressed protein had lost a critical functional domain (reviewed in Blaschuk et al., 1995). Alternatively, decreased adhesive function may result from mutations in the genes that encode the catenins. For example, mutations in β -catenin that disrupt the association of E-cadherin with α -catenin resulted in a nonadhesive phenotype (Oyama et al., 1994; Kawanishi et al., 1995). In other studies, it was shown that mutations in the gene that encodes α -catenin effectively inactivated E-cadherin function by disrupting association of the cadherin complex with the cytoskeleton (Hirano et al., 1992; Ewing et al., 1995; Nagafuchi et al., 1994). The result was the conversion of tumor cells to a metastatic phenotype. Thus, disrupting the function of the E-cadherin/catenin complex, independent of the mechanism of disruption, frequently results in the formation of invasive tumorigenic cells.

In this study, we explored the possibility that inappropriate expression of N-cadherin in a squamous epithelial cell that expresses E-cadherin and P-cadherin might also result in a cell with disrupted cell-cell adhesion and a more fibroblastic phenotype. We showed that a squamous cell carcinoma-derived cell line (UM-SCC-11B) expressed N-cadherin and that it had a scattered fibroblastic phenotype along with decreased expression of E-cadherin and P-cadherin. Transfection of this cell line with antisense N-cadherin resulted in reversion to a normal-appearing squamous epithelial cell with increased E-cadherin and P-cadherin expression. Interestingly, the levels of expression of N-cadherin and both E- and P-cadherin were inversely related to one another. In addition, we showed that squamous cell carcinomas did, on occasion, express N-cadherin in situ, and that those tumors that expressed N-cadherin were invasive.

Materials and Methods

Cell Culture

The human squamous cell carcinoma cultures UM-SCC-1 (SCC1), UM-SCC-11A (11A), and UM-SCC-11B (11B) were derived from surgical tumor specimens of histologically proven squamous cell carcinomas of the head and neck, and have been described previously (Baker, 1985). Cells were cultured in MEM supplemented with 10% FCS (Hyclone Laboratories, Logan, UT) and penicillin/streptomycin, as described (Kimmel and Carey, 1986).

The human fibroblast cell line WI38-VA13 was obtained from American Type Culture Collection (Rockville, MD) and maintained in DME (GIBCO BRL, Gaithersburg, MD), 10% FCS, and antibiotics (penicillin/streptomycin) at 50 U penicillin and 50 mg/liter streptomycin (GIBCO BRL).

Antibodies and Reagents

Unless otherwise stated, all reagents were from Sigma Chemical Co. (St. Louis, MO).

Rat monoclonal (E9) and rabbit polyclonal antibodies against human E-cadherin (Wheelock et al., 1987) and mAb against α -catenin (1G5) and β -catenin (12F7; Johnson et al., 1993), P-cadherin (6A9; Lewis et al., 1994), and N-cadherin (13A9; Sacco et al., 1995) have been described previously. The rabbit polyclonal antibody against α -catenin was purchased from Sigma. The mouse monoclonal antibody (HECD1) against human E-cadherin was a gift from Dr. Masatoshi Takeichi (The University of Kyoto, Kyoto, Japan). Tunicamycin was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA), and a stock solution (1 mg/ml) was prepared in DMSO and stored at -70° .

Molecular Constructs and Transfection

The full-length human N-cadherin clone in pBluescript was a gift from Dr. John Hemperly (Becton Dickinson Research Center, Research Triangle Park, NC; Reid and Hemperly, 1990). The expression vector pLKneo (Hirt et al., 1992) was a gift from Dr. Nicholas Fasel (University of Lausanne, Lausanne, Switzerland). For expression of full-length N-cadherin, the complete cDNA was inserted into pLKneo2. As a control for this experiment, the pLKneo2 vector without an insert was transfected into cells (mock transfection). For some transfection experiments, the neomycin gene in pLKneo was replaced with a slightly modified puromycin gene from pBSpac Δ p, a gift from Dr. Juan Ortín (Universidad Autónoma de Madrid, Madrid, Spain; de la Luna and Ortín, 1992). This vector is referred to as pLKpac. cDNAs inserted into the pLK series of vectors are under the control of the mouse mammary tumor virus promoter and are inducible by dexamethasone.

The production of antisense RNA against human N-cadherin used a plasmid, mU6, containing a modified human U6 snRNA gene kindly provided by Dr. Sarah Noonberg (University of California, San Francisco, CA; Noonberg et al., 1994). A hybrid U6 gene was created by replacing the XhoI/NsiI fragment in mU6 with antisense oligos spanning the start codon of human N-cadherin. The hybrid antisense construct was moved from mU6 into pBSpac Δ p as an EcoRI/BamHI fragment. The following two oligos were used to make the U6 hybrid gene: oligo-1, 5'-TCGAGTCCGCTATCCGGCACATGGAGGCGATGCA-3', and oligo-2, 5'-TCGCTCCATGTGCCGGATAGCGGGAC-3' (the start codon is underlined).

Cell cultures were transfected using a calcium phosphate kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Colonies of G-418 or puromycin-resistant cells were isolated and screened for expression of the transfected gene by Western blot analysis. Positive clones were further examined by immunofluorescence.

Microscopy

Cells were plated on glass coverslips in MEM with 10% FCS with or without 10^{-7} M dexamethasone. For morphological analysis of living cells, the coverslips were placed on a glass slide in mounting medium, and pictures were taken immediately using the 10 \times or 40 \times objective on an Axiophot microscope (Carl Zeiss Inc., Thornwood, NY).

For immunofluorescence, cells were grown on glass coverslips, fixed for 30 min in 1% paraformaldehyde buffered with HBSS containing 10 mM Hepes, pH 7.4, and permeabilized in methanol at -20° C for 3 min. Coverslips were blocked with 10% goat serum and 0.1 M glycine in PBS for 20 min and exposed to antibodies for 1 h, followed by species-specific FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Fluorescence was detected with a Zeiss Axiophot microscope equipped with epifluorescence. All pictures were taken using a 40 \times objective and T-Max 3200 film (Eastman Kodak Co., Rochester, NY).

Frozen tissue sections of biopsies of squamous cell carcinomas of the head and neck were collected from the tumor bank at the University of Michigan (Ann Arbor, MI). Tissue sections (8 μ m) were cut with a cryostat, placed onto poly-L-lysine-coated slides, and fixed in methanol at -20° C for 3 min. Sections were blocked by incubating in PBS containing 10% goat serum. Slides were stained with rabbit anti-E-cadherin and mouse anti-N-cadherin for 1 h, followed by FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Detergent Extraction of Cells

Monolayers of cells were washed with PBS at room temperature and were extracted on ice with 2 ml/75 cm² flask 10 mM Tris-acetate, pH 8.0, con-

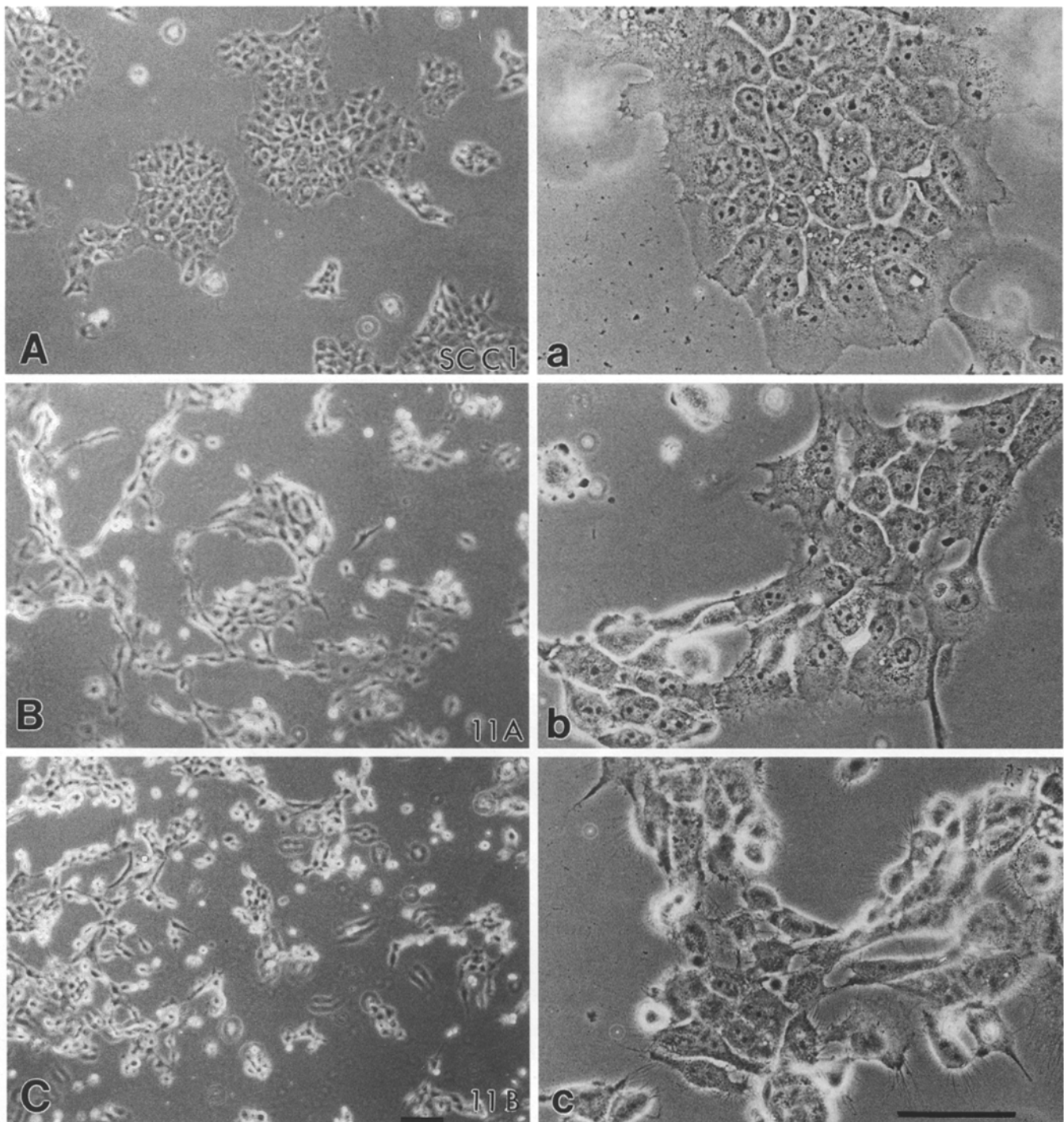


Figure 1. Morphological analysis of cell lines derived from squamous cell carcinomas. Living cells were photographed using the 10× (A, B and C) or 40× objective (a–c). A and a depict UM-SCC-1 (SCC1); B and b depict UM-SCC-11A (11A); C and c depict UM-SCC-11B (11B). Note the normal morphology of SCC1 and the progressively more scattered phenotype of 11A and 11B. Bars, 60 μ m.

taining 0.5% NP-40 (BDH Chemicals Ltd., Poole, UK), 1 mM EDTA, and 2 mM PMSF. Insoluble material was removed by centrifugation at 15,000 g for 15 min at 4°C.

Electrophoresis and Immunoblotting

SDS-PAGE was done as described previously (Johnson et al., 1993). Materials were from BioRad Laboratories (Richmond, CA). Molecular weight markers were from Sigma. SDS-PAGE-resolved proteins were transferred to nitrocellulose and immunoblotted as described (Knudsen

and Wheelock, 1992), using primary antibodies followed by alkaline phosphatase-conjugated anti-IgG (Promega, Madison, WI) with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates. Protein quantitation was done using the BioRad assay reagent according to the manufacturer's suggested protocol.

Immunoprecipitation

A 1-ml aliquot of cell extract was mixed with 100 μ l of monoclonal antibody supernatant at 4°C. After 30 min, 100 μ l of packed anti-mouse IgG-

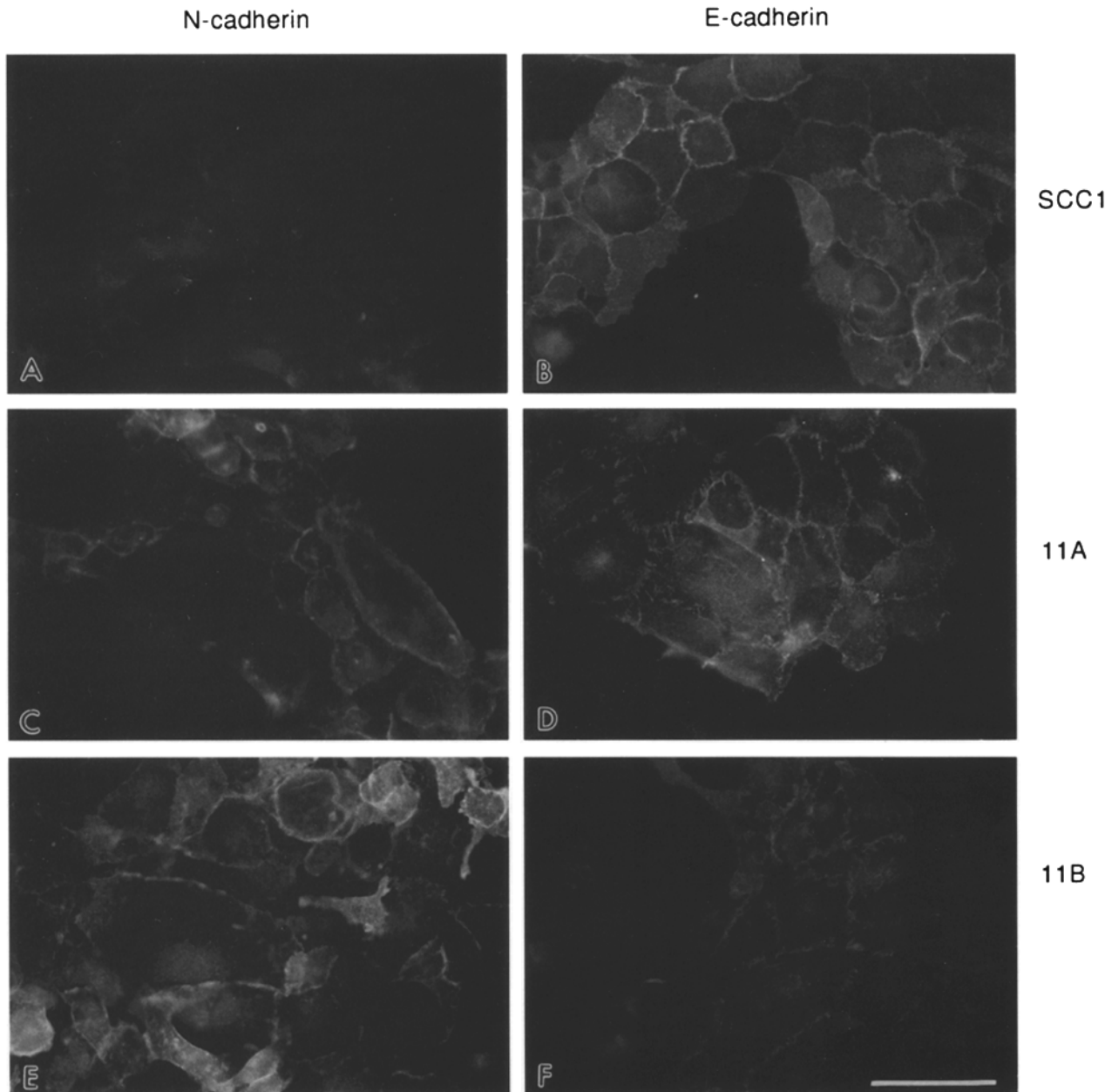


Figure 2. Expression of cadherins by squamous cell carcinoma–derived cell lines. Cells were grown on glass coverslips and processed for immunofluorescence using mAbs against E-cadherin (*E9*) or N-cadherin (*13A9*). (*A*, *C*, and *E*) The localization of N-cadherin. (*B*, *D*, and *F*) The localization of E-cadherin. Bar, 60 μm .

Sepharose (Organon Teknika-Cappel, Durham, NC) was added, and mixing was continued for 30 min. The Sepharose-bound immune complexes were washed five times with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween 20. The pellets were solubilized and resolved by SDS-PAGE.

Northern Blotting

Total RNA from SCC-1, 11A and 11B was prepared from 100-mm tissue culture dishes using RNA Isolator (Genosys Biotechnologies, Inc., Woodlands, TX) according to the manufacturer's instructions. 20 μg of total RNA from each cell line was electrophoresed on a 1% agarose formaldehyde gel and transferred to a nylon membrane. The membrane was probed with [^{32}P]dCTP-labeled 1.1-kb EcoRI fragment of human N-cadherin under high stringency conditions. Bands were visualized by autoradiography.

Results

Identification of Squamous Carcinoma Cells that Express N-Cadherin

Cell lines derived from squamous cell carcinomas of the head and neck were examined for cell–cell adhesive characteristics. Fig. 1 shows the morphology of representative cell lines. Panels *A* and *a* depict UM-SCC-1 (SCC1), a squamous carcinoma cell line from a tumor of the floor of the mouth. SCC1 had a typical squamous cell morphology with most cells exhibiting nearly continuous contact with surrounding cells. This cell line did not form tumors when injected into nude mice (Baker, 1985). Fig. 1, *B* and *b*, de-

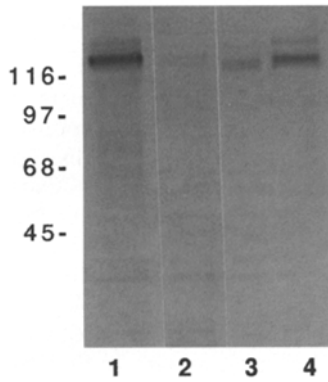


Figure 3. Western blot analysis of N-cadherin in squamous cell carcinoma-derived cell lines. Confluent monolayers of cells were extracted with NP-40, and equal amounts of protein from each extract were resolved by 7% SDS-PAGE, transblotted to nitrocellulose, and probed with mAbs against N-cadherin (13A9). Lane 1, control fibroblasts (WI38-VA13); lane 2, SCC1; lane 3, 11A; lane 4, 11B. Molecular weight markers are indicated.

pict UM-SCC-11A (11A), a cell line derived from a pre-treatment biopsy of squamous cell carcinoma of the larynx that was treated with chemotherapy but failed to respond. This cell line had a more scattered morphology with much less consistent cell-cell contacts. Fig. 1, C and c, depict UM-SCC-11B (11B), a cell line that was derived from the laryngectomy specimen of the persistent tumor of the larynx from which 11A was derived. 11B had an even more scattered phenotype than 11A. The 11B cell line was tumorigenic when tested in nude mice, while 11A grew poorly (Baker, 1985).

Normal squamous cells of the skin express E-cadherin and P-cadherin (Wheelock and Jensen, 1992), so we tested the three squamous cell carcinoma-derived cell lines for expression of these proteins. Fig. 2, B, D, and F, shows immunofluorescence staining for E-cadherin. SCC1 (Fig. 2 B) expressed levels of E-cadherin that were similar to what we typically see in normal squamous epithelial cells (Wheelock and Jensen, 1992). 11A (Fig. 2 D) and 11B (Fig. 2 F), however, expressed lower levels of E-cadherin. 11B, which had an even more scattered phenotype and was isolated from a postchemotherapy-recurrent tumor, expressed less E-cadherin than did 11A. Likewise, SCC1 expressed levels of P-cadherin that were similar to normal squamous epithelial cells, whereas 11A expressed less P-cadherin than SCC1 and 11B expressed less than 11A (data not shown).

Unexpectedly, the more scattered cell lines expressed N-cadherin, a cadherin that is normally not expressed by squamous epithelial cells. Fig. 2, A, C, and E, show immunofluorescence staining for N-cadherin in SCC1 (A), 11A (C), and 11B (E). In contrast to the E-cadherin expression, 11B cells expressed the highest levels of N-cadherin and SCC1 expressed the lowest levels of N-cadherin. Western blot analysis confirmed these findings. Fig. 3 compares the levels of N-cadherin expressed by the fibroblast cell lines WI38-VA13 (lane 1), SCC1 (lane 2), 11A (lane 3), and 11B (lane 4). Interestingly, the N-cadherin expressed by 11A and 11B migrated slightly faster on SDS-PAGE than did N-cadherin from the fibroblasts. However, Northern analysis showed that the messenger RNA encoding N-cadherin from 11B cells was the same size as the messenger RNA from the fibroblasts (data not shown). Thus, we suspected that N-cadherin in 11A and 11B cells was not correctly processed. We confirmed with tunicamycin experiments that N-cadherin in 11A and 11B cells was not properly glycosylated (not shown).

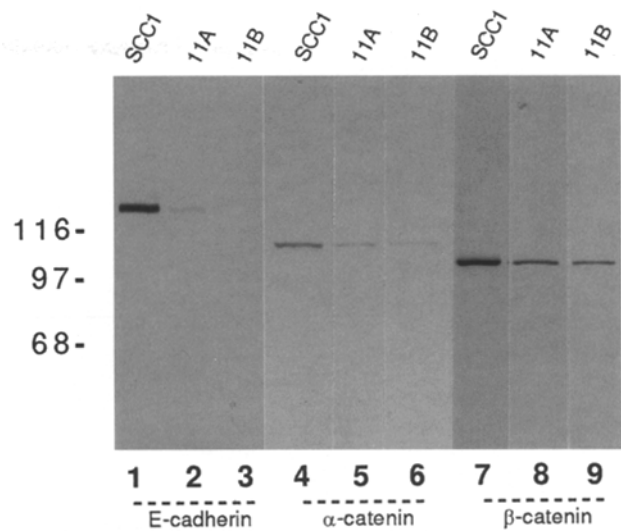


Figure 4. Western blot analysis of E-cadherin and catenins in squamous cell carcinoma-derived cell lines. Confluent monolayers of SCC1 cells (lanes 1, 4, and 7), 11A cells (lanes 2, 5, and 8), and 11B cells (lanes 3, 6, and 9) were extracted with NP-40, and equal amounts of protein from each extract were resolved by 7% SDS-PAGE, transblotted to nitrocellulose, and probed with mAbs against E-cadherin (E9, lanes 1-3), α -catenin (1G5, lanes 4-6), or β -catenin (12F7, lanes 7-9). Molecular weight markers are indicated.

To compare the expression levels of E-cadherin and the catenins in 11A and 11B with SCC1, we extracted cells with nonionic detergent, resolved equal amounts of protein from each cell line on SDS-PAGE, and immunoblotted with antibodies against the cadherins and the catenins. Fig. 4 shows that SCC1 (lane 1) expressed significantly more E-cadherin than did either 11A (lane 2) or 11B (lane 3). Although we could see staining for E-cadherin by immunofluorescence in all three cell lines, it was not detectable in 11B by our immunoblotting technique. When we assayed for P-cadherin, we found similar results; SCC1 expressed high levels, while 11A and 11B expressed much lower levels (data not shown). α -Catenin and β -catenin were more abundant in SCC1 (lanes 4 and 7, respectively), but were easily detectable in both 11A (lanes 5 and 8) and 11B (lanes 6 and 9).

We were equally interested in whether or not the cadherins (both the normally expressed E- and P-cadherins, as well as the aberrantly expressed N-cadherin) were complexed with catenins in each of the SCC cell lines. To address this question, we immunoprecipitated the cadherins, resolved the immunoprecipitation reactions by SDS-PAGE, and immunoblotted with a mixture of anticadherin, anti- α -catenin and anti- β -catenin. Fig. 5 shows that both α -catenin and β -catenin coimmunoprecipitated with E-cadherin from SCC1 (lane 1), 11A (lane 2), and 11B (lane 3). Although 11B had much less E-cadherin than the other cell lines, the E-cadherin that was expressed was associated with catenins. Likewise, N-cadherin was complexed with catenins in all three cell lines (Fig. 5, lanes 4-6). As the levels of expression of N-cadherin increased in the squamous cell carcinomas, the levels of both E-cadherin and P-cadherin decreased. In all three cell lines, however, the

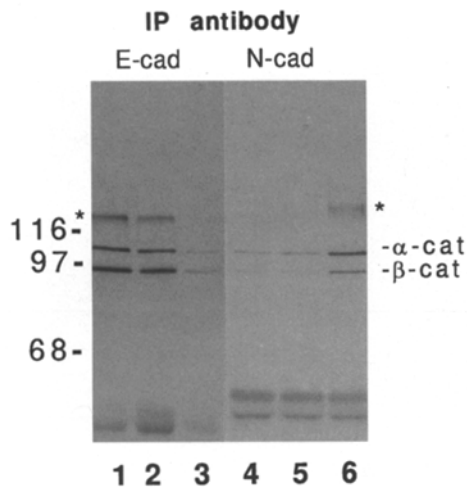


Figure 5. E-cadherin and N-cadherin are associated with catenins in the SCC cell lines. Confluent monolayers of SCC1 cells (lanes 1 and 4), 11A cells (lanes 2 and 5) and 11B cells (lanes 3 and 6) were extracted with NP-40, and equal amounts of protein from each extract were immunoprecipitated with mAbs against E-cadherin (HECD-1, lanes 1–3) or N-cadherin (13A9, lanes 4–6). The immunoprecipitation reactions were resolved by 7% SDS-PAGE, transblotted to nitrocellulose, and probed with a cocktail of mAbs against α -catenin (α -cat), β -catenin (β -cat), and cadherin (E-cadherin in lanes 1–3 and N-cadherin in lanes 4–6; the cadherin is pointed out by an asterisk). In all cases, α -catenin, β -catenin, and cadherin were in the immunoprecipitation reactions. The levels of cadherin and catenin varied according to how much cadherin was expressed by the cell line. The IgG heavy chains from the immunoprecipitation reaction are visible at the bottom of the gel. Molecular weight markers are indicated. *IP*, immunoprecipitation.

cadherin was complexed with catenins, indicating that it was likely to be functional.

N-Cadherin Is Expressed by Some Squamous Cell Carcinomas In Vivo

It was important to determine whether or not epithelial cells residing within tumors actually expressed N-cadherin or if the expression of N-cadherin was an artifact of culture. Our prediction was that if N-cadherin was being inappropriately turned on in the tumor, we would be able to identify epithelial cells that expressed N-cadherin within the tissue. To address this, we surveyed a bank of 47 frozen squamous cell carcinomas from patients with cancer of the head and neck for coexpression of E-cadherin (as a marker for epithelial cells) and N-cadherin. Tumor sections were costained with a rabbit polyclonal antiserum against E-cadherin and a mouse mAb against N-cadherin. The rabbit and mouse antibodies were detected with secondary anti-IgG labeled with FITC and rhodamine, respectively. In each case, the squamous cells were uniformly positive for E-cadherin, as expected for epithelial cells. In eight of the tumors, small islands of cells that were positive for N-cadherin were observed. Fig. 6 presents two representative examples of our survey: *A* and *C* show the staining for E-cadherin; *B* and *D* show colocalization of N-cadherin in the sections depicted in *A* and *C*, respectively. N-cadherin-positive cells are pointed out by arrows. Analysis of the histories involved in this study re-

vealed that all of the N-cadherin positive tumors showed evidence of invasion.

Transfection of N-Cadherin into SCC1 Cells Results in a Less-adhesive Phenotype

An important question we wished to address was whether or not expression of N-cadherin was likely to be responsible for the scattered phenotype of 11A and 11B cells. To address this question, we transfected full-length N-cadherin under the control of the dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter into SCC1 cells. N-cadherin-expressing clones were selected and observed for morphological characteristics. Fig. 7 compares N-cadherin-transfected SCC1 cells (*B*, *b*, *C*, and *c*) with mock-transfected SCC1 cells (*A* and *a*). In the absence of dexamethasone the transfected cells displayed a squamous cell morphology, as did the mock-transfected cells (*B* with *A*) or the parent untransfected SCC1 cells (see Fig. 1 *A*). When expression was induced with dexamethasone for 72 h, however, a distinct morphological change became apparent (compare *C* and *c* with *A* and *a*); the cells began to scatter and to acquire a phenotype more like 11A and 11B (see Fig. 1, *B* and *C*). Fig. 8 shows that the level of N-cadherin expression increased when the transfected cells were induced with dexamethasone, and that the level of E-cadherin decreased concomitantly with the increase in N-cadherin. For this figure, we deliberately chose to photograph fields that included cells with good cell-cell contact to compare the expression of the cadherins. Overall, the cells looked identical to those shown in Fig. 7. Fig. 8, *A* and *B*, show immunofluorescence staining for N-cadherin in uninduced (*A*) and induced (*B*) transfectants. Fig. 8, *C* and *D*, show the expression of E-cadherin when the cells are uninduced or induced, respectively. Dexamethasone had no effect on the expression of E-cadherin or N-cadherin in control mock-transfected cells or in SCC1 cells (not shown).

Equal amounts of protein from uninduced and induced cultures were immunoprecipitated with antibodies against E-cadherin or N-cadherin to analyze the levels of expression of the cadherins and to determine whether or not the cadherins were complexed with catenins in the transfected cells. Fig. 9, lanes 1 and 2, show that the uninduced transfectant (lane 1) expressed more E-cadherin than the induced transfectant (lane 2), and that the E-cadherin was associated with catenins in both cases. Lanes 3 and 4 indicate a significant increase in the expression of N-cadherin in the induced transfectant (lane 4) when compared to the uninduced cells (lane 3). As in the 11B cells, the transfected N-cadherin was associated with both α -catenin and β -catenin. Unlike the 11B cells, N-cadherin appeared to be properly processed in transfected SCC1 cells since it comigrated on SDS-PAGE with N-cadherin from fibroblasts (not shown). Thus, the ability of N-cadherin to induce a scattered phenotype in squamous epithelial cells was not caused by its improper processing.

The Scattered Phenotype of UM-SSC-11B Can be Reversed by Transfection of N-Cadherin Antisense cDNA

The experiments discussed above indicate that expression

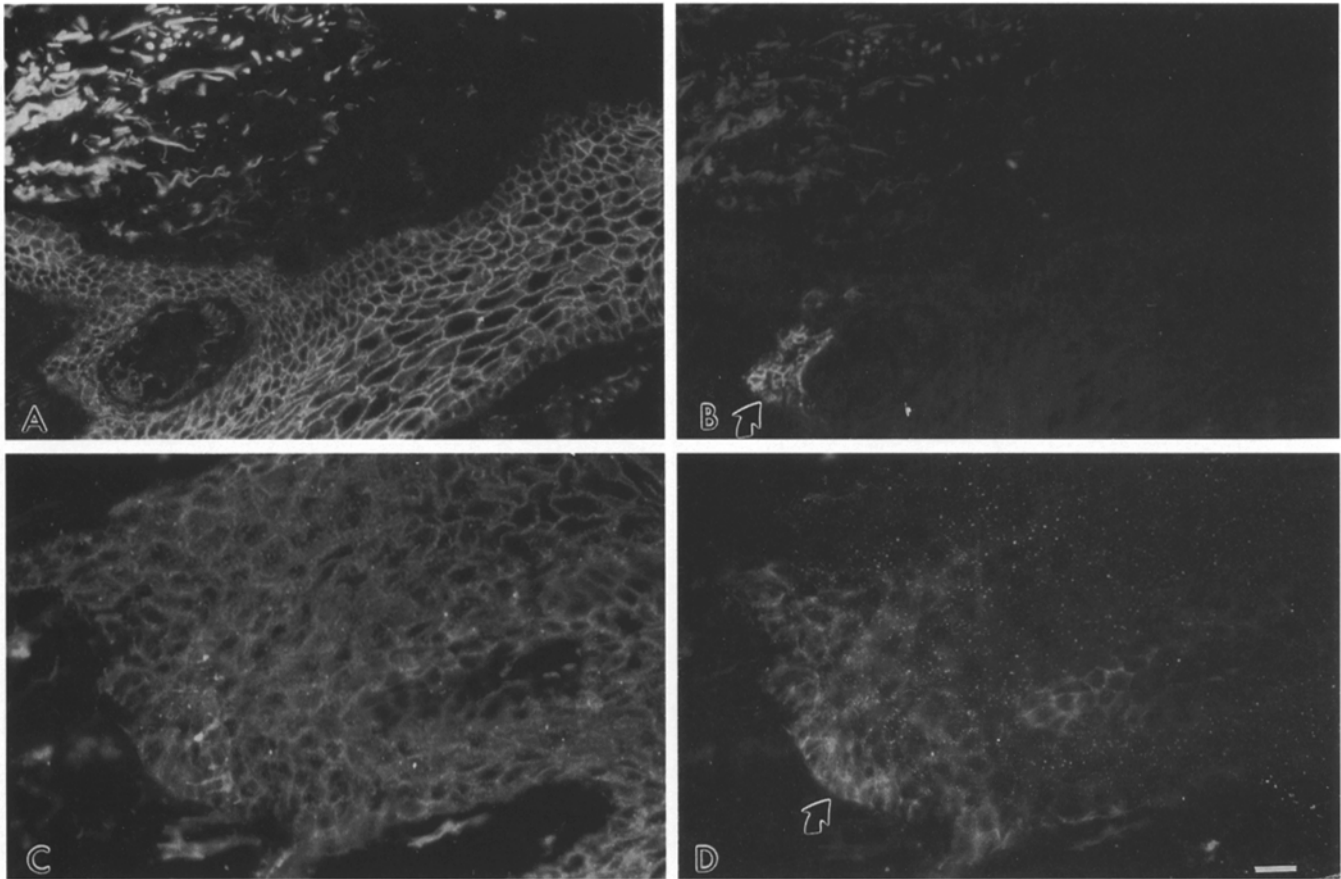


Figure 6. Expression of N-cadherin by squamous cell carcinomas of the head and neck. Frozen tissue biopsies were sectioned onto poly-L-lysine-coated slides and processed for immunofluorescence. Slides were stained with rabbit anti-E-cadherin plus mouse anti-N-cadherin for 1 h, followed by FITC-conjugated anti-rabbit IgG plus rhodamine-conjugated anti-mouse IgG. Pictures were taken using the 40 \times objective. (A and C) The localization of E-cadherin in two different tumors. (B and D) The colocalization of N-cadherin in small islands of cells, which are pointed out by arrows. The bright staining in the upper left hand corner of A (slightly visible in B also) was caused by autofluorescence (more prevalent in the FITC channel than in the rhodamine channel), and was seen even in unstained sections. Bar, 60 μ m.

of N-cadherin by squamous epithelial cells can result in a more scattered phenotype. However, they do not tell us specifically that the phenotype of 11B results from the expression of N-cadherin. To address this question, we transfected antisense N-cadherin into 11B to decrease the expression of N-cadherin by these cells. To obtain high levels of expression of the antisense construct, we inserted antisense oligos into a modified U6 gene; the promoter of U6 is constitutively active (Noonberg et al., 1994). Fig. 10 shows phase contrast micrographs of the transfected cells. When compared to the untransfected 11B cells (see Figs. 1 and 7) the transfected cells had undergone a dramatic change in morphology. They appeared to have much closer contacts with one another and a more cohesive, less scattered appearance. Their appearance now closely resembled that of SCC1 cells (see Figs. 1 and 7). Western blot analysis (Fig. 11) showed that the antisense-transfected 11B cells expressed less N-cadherin (compare lane 4 with lane 3) and more E-cadherin (compare lane 2 with lane 1) than did the parent 11B cells. Immunofluorescence microscopy confirmed these data. Fig. 12 A shows that N-cadherin was barely detectable in the antisense-transfected 11B cells. Fig. 12 B shows that E-cadherin expres-

sion was upregulated in the antisense-transfected 11B cells, and that it was localized at cell-cell borders. These results were not caused by clonal variation in the 11B cell line, since we obtained identical results with several different clones.

Thus, we have presented data that strongly suggest that inappropriate expression of a nonepithelial cadherin in a squamous epithelial cell line resulted in a cell with a more scattered and less adhesive phenotype. In addition, we have shown that N-cadherin was, on occasion, expressed by epithelial cells in squamous cell carcinomas of the head and neck, and that there was a high probability of invasion in those tumors with N-cadherin-positive cells. Finally, we have shown that a scattered squamous cell carcinoma can revert to a cell with a classical epithelial morphology when transfected with antisense to N-cadherin, indicating that the scattered phenotype is a direct result of expression of N-cadherin. Interestingly, E-cadherin and N-cadherin expression were reciprocally coregulated in these cells.

Discussion

In the present study, we have shown that inappropriate ex-

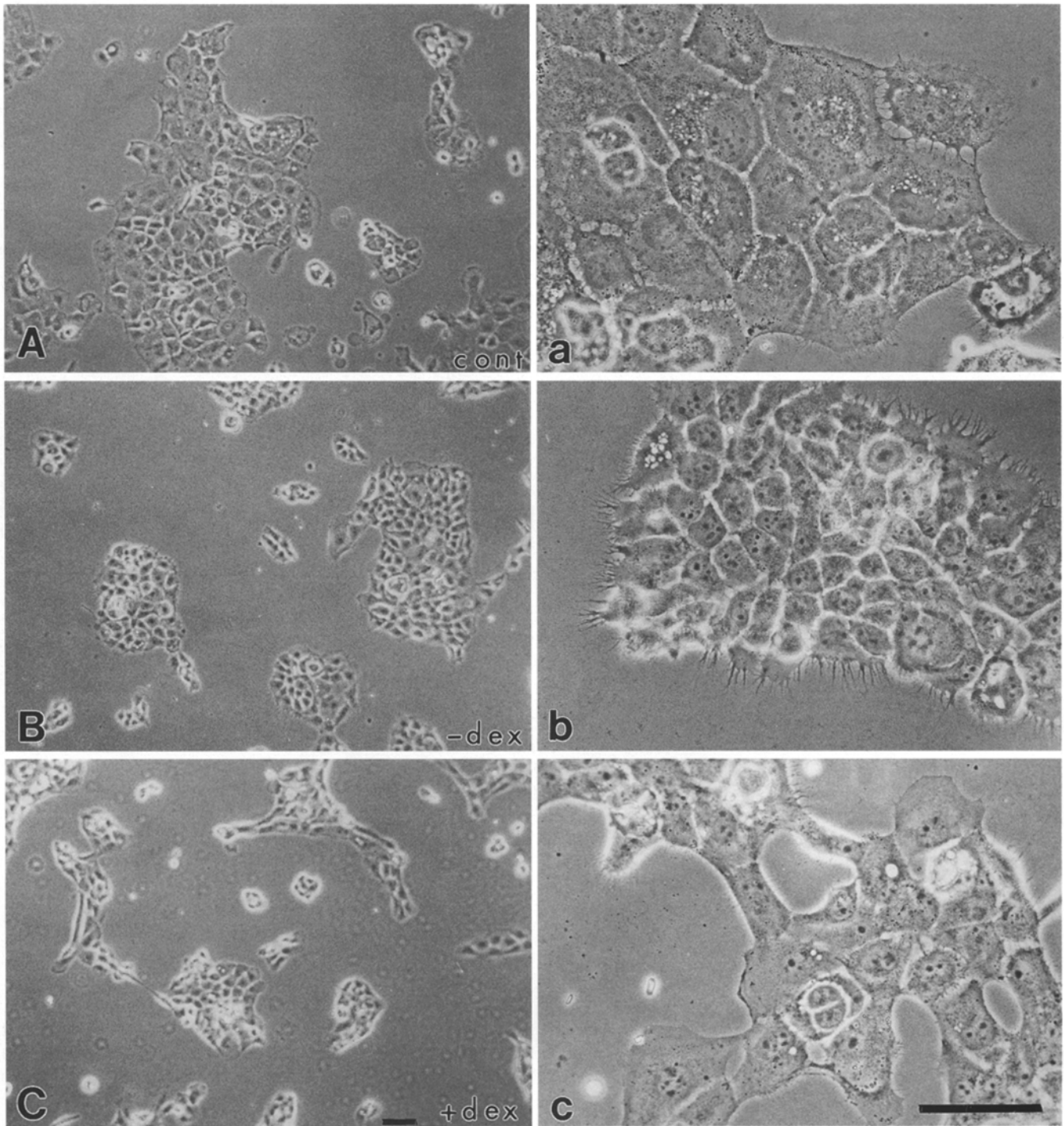


Figure 7. Transfection of SCC1 cells with N-cadherin resulted in a more scattered phenotype. SCC1 cells were transfected with N-cadherin under the control of a dexamethasone-inducible promoter. Living cells were photographed using the 10 \times (A–C) or 40 \times (a–c) objective. A and a show SCC1 cells mock transfected with the vector without an insert (*cont*) treated with dexamethasone for 72 h as controls for morphological changes. B, b, C, and c show the transfected cells without (–dex) and with (+dex) dexamethasone for 72 h. Note the change in morphology when the transfected cells are induced with dexamethasone. Bars, 60 μ m.

pression of N-cadherin by epithelial cells can result in cells with the more scattered, less adhesive phenotype, which is typical of invasive tumor cells. Numerous studies have shown that disruption of the E-cadherin/catenin complex in epithelial cells results in biologically aggressive tumor cells, implicating E-cadherin as the product of a tumor

suppressor gene (Vleminckx et al., 1991; Navarro et al., 1991; Frixen et al., 1991; Umbas et al., 1992; Schipper et al., 1991; Pierceall et al., 1995; Bex et al., 1995; reviewed in Takeichi, 1993). Such studies have shown that mutations or deletions that affect the function of the cadherin or one of the catenins contribute to the tumorigenic phenotype.

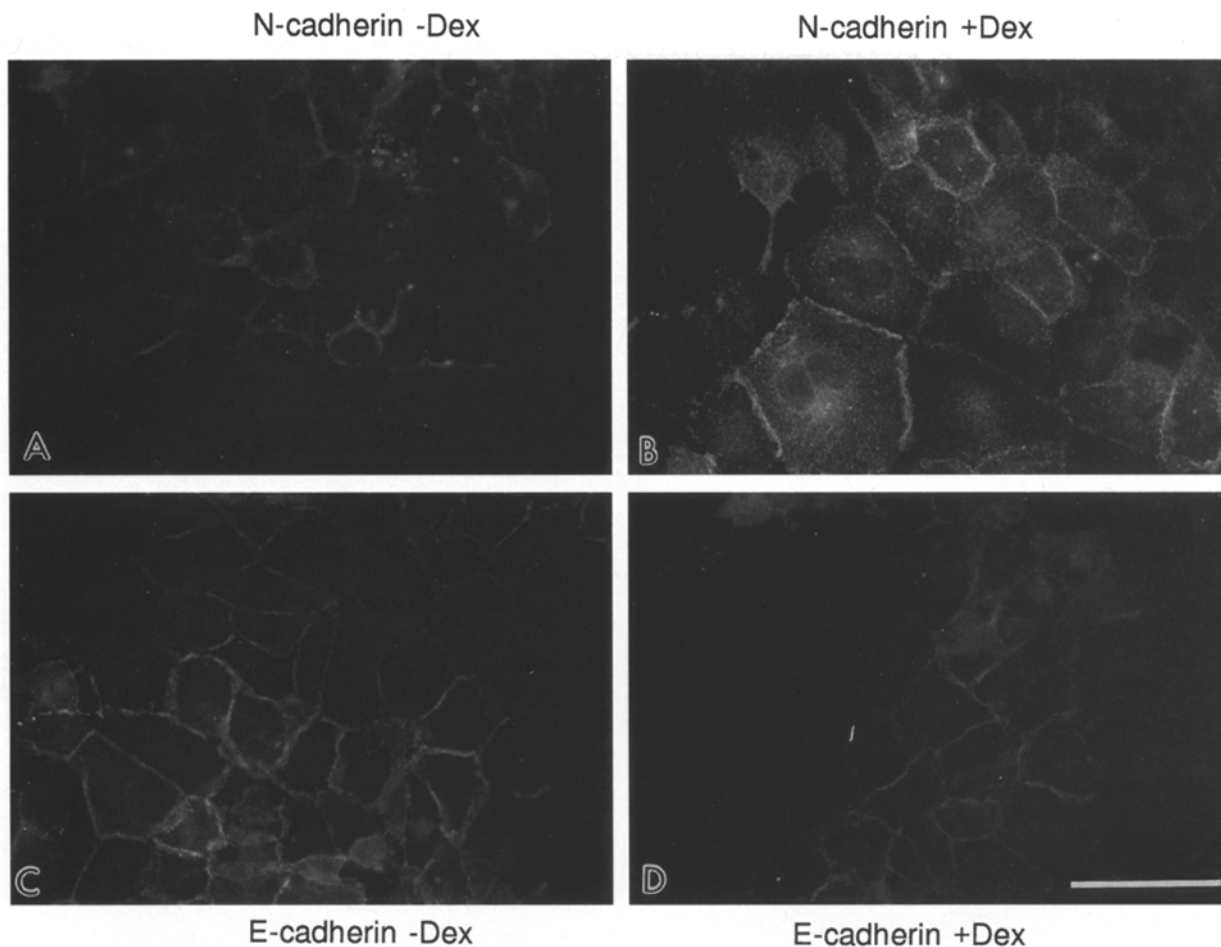


Figure 8. Expression of E-cadherin and N-cadherin in SCC1 cells transfected with N-cadherin. Transfected cells were grown on glass coverslips without (*A* and *C*) or with (*B* and *D*) dexamethasone, and were processed for immunofluorescence using mAbs against N-cadherin (13A9, *A* and *B*) or E-cadherin (E9, *C* and *D*). Note the increase in N-cadherin and concomitant decrease in E-cadherin expression when the cells are induced with dexamethasone. Bar, 60 μ m.

Regardless of the mechanism of disruption, whether it is caused by a deletion in α -catenin or β -catenin such that the cadherin complex cannot interact with actin filaments, or by a mutation or deletion in the cadherin itself so that it cannot function as an adhesion molecule, the result is similar; the cells are more likely to become invasive. It is unexpected and interesting that a similar phenotype is seen in cells that express an inappropriate cadherin, perhaps suggesting that an epithelial/mesenchyme transition reminiscent of those seen during some developmental processes may be inadvertently activated in these cells.

The cadherins are members of a large family of related proteins. Epithelial cells express primarily E-cadherin alone or E-cadherin and P-cadherin, although other cadherins have been demonstrated in some epithelial cells. Neurons, mesothelial cells, muscle, lens epithelial cells, and fibroblasts express N-cadherin. N-cadherin can function as a respectable adhesion molecule; for example, in cardiac muscle, N-cadherin plays an essential role in the formation of strong cell-cell contacts that allow the cells to beat coordinately (Volk and Geiger, 1984; Peralta Soler and Knudsen, 1994). In addition, tissues such as the mesothelium and the lens use N-cadherin to form contacts that very much resemble the E-cadherin-mediated con-

tacts of epithelial cells (Volk and Geiger, 1984; Peralta Soler et al., 1995). However, N-cadherin is also expressed by cells such as fibroblasts that do not display close cell-cell contacts, but rather, act as solitary cells most of the time. Fibroblasts and cardiac muscle cells express approximately equal levels of N-cadherin, and the N-cadherin/catenin complex in these two cell types appears to be identical (Wheelock and Knudsen, 1991; Knudsen et al., 1995); however, the adhesive characteristics of these two cell types is quite different. It is not clear why expression of N-cadherin by squamous epithelial cells is not compatible with the typical epithelial cell morphology. One could argue that the dramatic difference in the morphology of epithelial cells and fibroblasts is the presence of desmosomes. Theoretically, squamous epithelial cells should be able to make desmosomes, even when E-cadherin is replaced by N-cadherin, since cardiac muscle cells that express N-cadherin make desmosomes. Our hypothesis is that it is a difference in signaling capabilities of the individual cadherins rather than the physical ability of the cadherin to mediate adhesion that contributes to the difference in phenotype when squamous epithelial cells express N-cadherin rather than E-cadherin. We are currently addressing this question using chimeric E-cadherin/N-cadherin molecules.

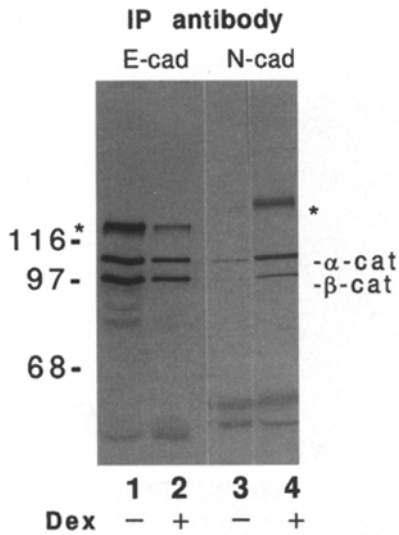


Figure 9. Catenins were associated with N-cadherin in the transfected SCC1 cells. Confluent monolayers of SCC1 cells transfected with N-cadherin, either uninduced (-) or induced (+), were extracted with NP-40, and equal amounts of protein from each extract were immunoprecipitated with mAbs against E-cadherin (HECD-1, lanes 1 and 2) or N-cadherin (13A9, lanes 3 and 4). The immunoprecipitation reactions were resolved by 7% SDS-PAGE, transblotted to nitrocellulose, and probed with a cocktail of mAbs against α -catenin (α -cat), β -catenin (β -cat), and cadherin (*E-cad* in lanes 1 and 2 and *N-cad* in lanes 3 and 4; the cadherin is pointed out by an asterisk). In all cases, α -catenin, β -catenin, and cadherin were in the immunoprecipitation reactions. When N-cadherin expression was induced (lanes 2 and 4), the level of N-cadherin increased and the level of E-cadherin decreased. Molecular weight markers are indicated.

We became interested in cells derived from squamous cell carcinomas when we discovered that some squamous cell carcinoma-derived cell lines expressed N-cadherin. In fact, even the normal-appearing cells with high levels of E-cadherin and P-cadherin expressed a small amount of N-cadherin. It has not been determined whether normal squamous epithelial cells express small amounts of N-cadherin. One possibility is that squamous epithelial cells always express trace levels of N-cadherin, and the tumor cells have inadvertently turned on higher levels of expression that resulted in the observed phenotype. It appears that the scattered phenotype is directly caused by increased N-cadherin expression because we could mimic it in the laboratory simply by transfecting N-cadherin into normal-appearing squamous epithelial cells. Even stronger evidence for N-cadherin being the cause of the scattered phenotype was obtained when the phenotype of 11B cells reverted to a more normal adhesive squamous cell appearance after transfection with an N-cadherin antisense construction. It is not known whether the change in phenotype of the N-cadherin-expressing cells is caused directly by N-cadherin or by the resultant decrease in E-cadherin expression.

It appears that E-cadherin and N-cadherin levels are reciprocally regulated in squamous epithelial cells. When N-cadherin was ectopically expressed in SCC1 cells, the levels of E-cadherin decreased; when N-cadherin expres-

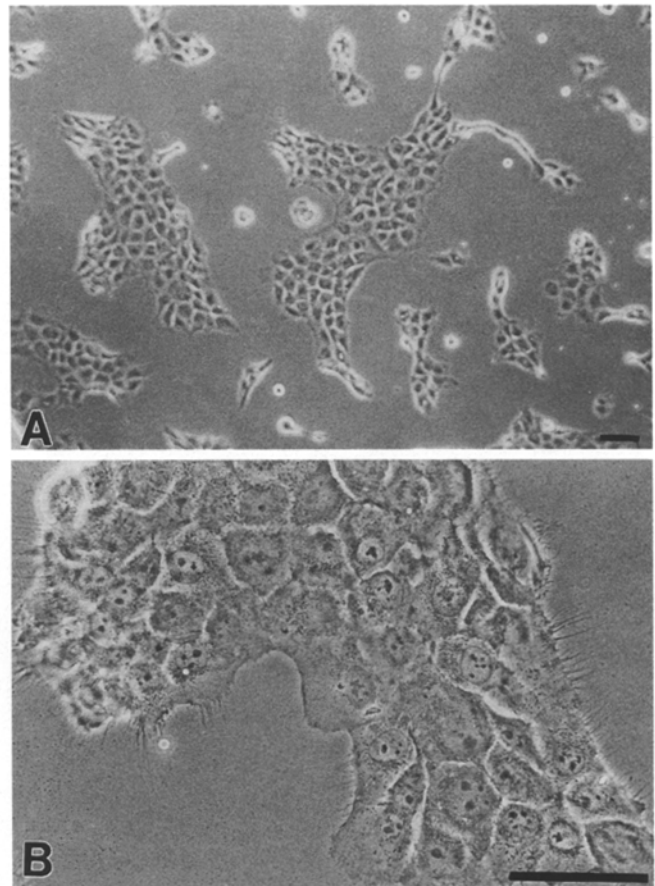


Figure 10. Transfection of 11B with antisense N-cadherin resulted in a cell line with a normal squamous epithelial cell morphology. The 11B cell line was transfected with antisense N-cadherin, and living cells were photographed using the 10 \times objective (A) or the 40 \times objective (B). Note the normal morphology of the transfected cells. Bars, 60 μ m.

sion in 11B was decreased by introduction of an antisense construct, the levels of E-cadherin and P-cadherin increased dramatically. The mechanism of this coregulation is not understood. However, there are several examples of coregulation of cadherin levels during development. For example, in two major morphogenetic events, gastrulation and neurulation, cells that segregate from the ectoderm gradually cease to express E-cadherin and begin to express N-cadherin (Takeichi, 1987). Recently, it was suggested that reciprocal expression of E-cadherin and cadherin-11 may play a role in trophoblast-endometrial cell interactions during implantation of the mammalian embryo (MacCallman et al., 1996). Perhaps expression of N-cadherin by squamous cell carcinomas facilitates cells leaving the site of the tumor and invading the underlying stroma, where they are compatible with stromal N-cadherin-expressing cells.

It will be interesting to examine the SCC1 and 11B cells along with the transfectants for expression of other adhesion molecules. For example, several recent studies have suggested that integrin expression and cadherin expression may be coregulated (Hodivala and Watt, 1994; Burdsal et al., 1993) in some systems. We are currently investigating changes of expression of integrins by SCC1 and

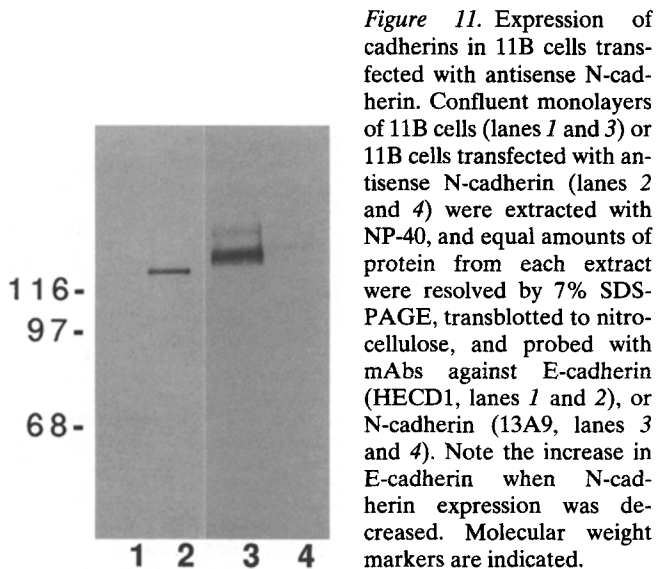


Figure 11. Expression of cadherins in 11B cells transfected with antisense N-cadherin. Confluent monolayers of 11B cells (lanes 1 and 3) or 11B cells transfected with antisense N-cadherin (lanes 2 and 4) were extracted with NP-40, and equal amounts of protein from each extract were resolved by 7% SDS-PAGE, transblotted to nitrocellulose, and probed with mAbs against E-cadherin (HECD1, lanes 1 and 2), or N-cadherin (13A9, lanes 3 and 4). Note the increase in E-cadherin when N-cadherin expression was decreased. Molecular weight markers are indicated.

SCC11B upon transfection of N-cadherin and N-cadherin antisense constructs, respectively.

In addition to the interesting cell biology, there is also significant clinical relevance to our findings. All of the tu-

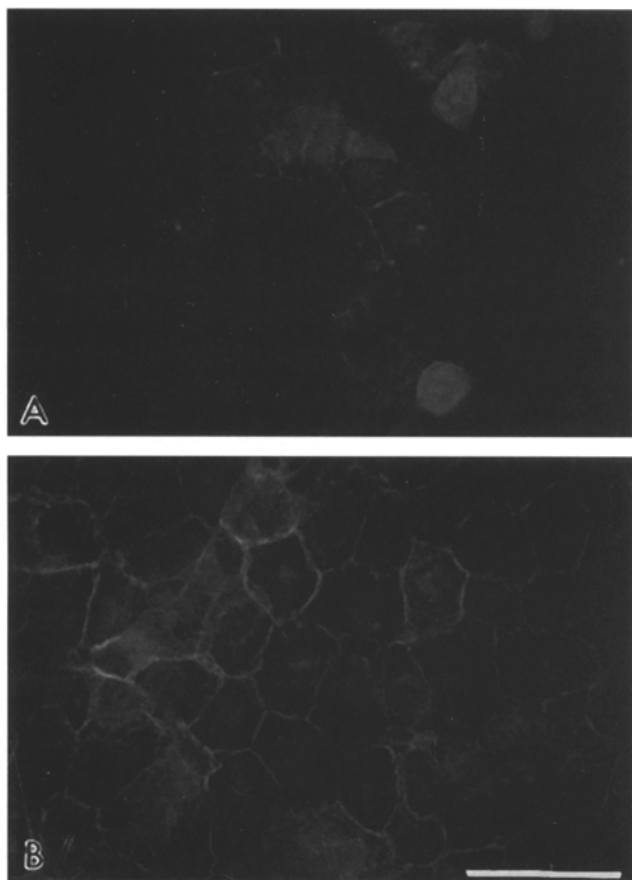


Figure 12. Localization of cadherins in 11B cells transfected with antisense N-cadherin. Transfected 11B cells were grown on glass coverslips and processed for immunofluorescence using mAbs against N-cadherin (13A9, A) or E-cadherin (E9, B). Note the increase in E-cadherin and the concomitant decrease in N-cadherin expression. Bar, 60 μ m.

mors that included N-cadherin-positive colonies of cells were found to subsequently invade the surrounding tissue. In addition, the SCC1 cell line failed to produce tumors in nude mice, 11A grew poorly in nude mice, and 11B grew well in vivo (Baker, 1985). Together, these data suggest that there is a correlation between expression of N-cadherin by squamous epithelial cells and the ability of these cells to form tumors in nude mice and to invade the surrounding tissue. We would like to suggest that expression of N-cadherin by squamous epithelial cells may be an indicator of a potentially aggressive tumor. However, a much larger clinical study is needed to confirm this. We would also like to suggest that in addition to examining tumor cells for mutations in the adhesion molecules they would normally express, it may also be important to examine them for inappropriate expression of other adhesion molecules.

It is interesting that E-cadherin expression is not permanently lost in E-cadherin-negative squamous cell carcinomas, since blocking N-cadherin expression upregulated E-cadherin. This would suggest that further studies are needed to elucidate the mechanisms that regulate cadherin expression and the switch from one cadherin. Elucidation of physiological or environmental molecules that may regulate this switch could provide clues to effective treatment or prevention of squamous cell carcinomas.

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