E-Cadherin-mediated Cell–Cell Adhesion Prevents Invasiveness of Human Carcinoma Cells

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Abstract. The ability of carcinomas to invade and to metastasize largely depends on the degree of epithelial differentiation within the tumors, i.e., poorly differentiated being more invasive than well-differentiated carcinomas. Here we confirmed this correlation by examining various human cell lines derived from bladder, breast, lung, and pancreas carcinomas. We found that carcinoma cell lines with an epithelioid phenotype were noninvasive and expressed the epithelium-specific cell–cell adhesion molecule E-cadherin (also known as Arc-1, uvomorulin, and cell-CAM 120/80), as visualized by immunofluorescence microscopy and by Western and Northern blotting, whereas carcinoma cell lines with a fibroblastoid phenotype were invasive and had lost E-cadherin expression. Invasiveness of these latter cells could be prevented by transfection with E-cadherin cDNA and was again induced by treatment of the transfected cells with anti–E-cadherin mAbs. These findings indicate that the selective loss of E-cadherin expression can generate dedifferentiation and invasiveness of human carcinoma cells, and they suggest further that E-cadherin acts as an invasion suppressor.

OVER 90% of the human tumors are carcinomas; in these, transformed epithelial cells grow in an uncontrolled fashion, break through the basement membrane, and invade the underlying mesenchyme. Carcinomas can be subdivided by morphological and functional criteria: (*a*) well-differentiated carcinomas largely retain epithelial tissue structures, they show well-developed intercellular junctions, and they are generally weakly invasive, and (*b*) poorly differentiated carcinomas are characterized by an amorphous tissue structure, they have fewer cell-to-cell junctions, and they are more invasive (Weinstein et al., 1976; Gabbert et al., 1985). It has also been shown that the state of differentiation and the degree of invasiveness of carcinomas can determine cancer prognosis. For instance, 80% of patients with well-differentiated colorectal carcinomas survive five and more years, in contrast to only 25% of patients with diagnosis of poorly differentiated colorectal carcinomas (Morson and Dawson, 1979).

These morphological and functional characteristics of carcinomas have been recognized years ago; the underlying molecular basis, however, is only presently being investigated. Various oncogenes have been found to be implicated in the genesis of human carcinomas, e.g., the Ki-*ras* oncogene (Bos et al., 1987; Forrester et al., 1987; Almoguera et al., 1988), and the HER-2/*neu* protooncogene (Slamon et al., 1987, 1989). Various tumor suppressor genes are mutated or deleted in human carcinomas, e.g., the retinoblastoma susceptibility gene (Harbour et al., 1988; Lee et al., 1988; Bookstein et al., 1990), the Wilms tumor gene (Habor et al., 1990; Gessler et al., 1990), the p53 gene (Takahashi et al., 1989; Nigro et al., 1989; Tsai et al., 1990), the deleted in colon carcinomas (DCC) gene (Fearon et al., 1990), a gene on chromosome 5q (Bodmer et al., 1987; Leppert et al., 1987), and a gene (or genes) on chromosome 3p (Kok et al., 1987; Zbar et al., 1987; Kovacs et al., 1988).

Keratins and desmosomal proteins allow the identification and classification of epithelial tumors and can also be used for tracing back the origin of metastatic carcinomas (Osborn et al., 1977; Gabbiani et al., 1981; Osborn and Weber, 1983; Cooper et al., 1985; Moll et al., 1986). Changes in the keratin expression pattern which parallel dedifferentiation have recently been observed in bladder carcinomas (Moll et al., 1988). The carcinoembryonic antigen (CEA) has been found to be a valuable indicator of carcinomas of the gastrointestinal tract (Mentges, 1987; Benchimol et al., 1989; Daneker et al., 1989), the expression of urokinase and its receptor is restricted to dedifferentiated colon carcinoma cell lines (Boyd et al., 1988), and the estrogen receptor represents a useful marker for differentiated breast carcinomas (Engel and Young, 1978; Sluyser, 1990).

Our laboratory has studied epithelial differentiation and invasion with respect to the expression and function of the epithelium-specific cell–cell adhesion molecule E-cadherin, which various investigators have differently named Arc-1 (Imhof et al., 1983; Behrens et al., 1985), uvomorulin (Hyafil et al., 1981; Vestweber and Kemler, 1984), L-CAM (Gallin et al., 1983), E-cadherin (Shirayoshi et al., 1983), and cell-CAM 120/80 (Damsky et al., 1983). We have demonstrated that nontransformed MDCK epithelial cells

1. *Abbreviations used in this paper*: CEA, carcinoembryonic antigen; DCC, deleted in colon carcinomas; DFKZ, German Cancer Research Center.

acquire invasive properties when intercellular adhesion is specifically inhibited by the addition of antibodies against E-cadherin; the separated cells then assume a fibroblast-like, i.e., dedifferentiated morphology, and invade collagen gels and embryonal heart tissue. Furthermore, MDCK cells transformed with Harvey and Moloney sarcoma viruses were found to be constitutively fibroblast-like and invasive, and they do not express E-cadherin. These data suggested that the loss of adhesive function of E-cadherin is a critical step in the promotion of epithelial cells to a dedifferentiated and invasive, i.e., malignant stage (Behrens et al., 1985, 1989).

The cell-adhesion molecule E-cadherin represents a 120-kD cell surface glycoprotein, of which extracellularly an 80-kD soluble tryptic fragment can be released in the presence of Ca^{2+} (Hyafil et al., 1981; Damsky et al., 1983; Gallin et al., 1983; Peyrieras et al., 1983). In early mouse development E-cadherin functions as an adhesion component during compaction of blastomeres (Hyafil et al., 1981; Damsky et al., 1983; Shirayoshi et al., 1983; Vestweber and Kemler, 1984), at later stages it is confined to epithelia originating from ecto-, meso-, and endodermal tissue (Edelman et al., 1983; Damsky et al., 1983; Imhof et al., 1983; Vestweber and Kemler, 1984; Behrens et al., 1985). In the epithelium of the small intestine, E-cadherin is enriched in the adherens junctions, in other epithelia it is present at the lateral cell surfaces (Damsky et al., 1983; Boller et al., 1985; Behrens et al., 1985). The full cDNA of the mouse molecule has recently been cloned; it codes for a signal peptide at the NH_2 terminus, a large extracellular domain with four repeats, a single transmembrane region, and a 15-kD cytoplasmic tail (Nagafuchi et al., 1987; Ringwald et al., 1987). After transfection of the cDNA into fibroblasts, functional Ca^{2+}-dependent contacts between the cells could be generated (Nagafuchi et al., 1987; Mege et al., 1988; Ozawa et al., 1989). E-cadherin belongs to a gene family; the closest relatives are N-cadherin (expressed in neural and muscle cells, Hatta et al., 1985, 1988) and P-cadherin (originally identified in mouse placenta but has also been found in a restricted set of human epithelia; Shimoyama et al., 1989*a*, *b*), a further relative is desmoglein (Koch et al., 1990). E-, N-, and P-cadherin exhibit a unique spatiotemporal expression pattern during embryogenesis, and cells expressing different cadherins sort out in vitro, suggesting that these adhesion molecules play a key role during morphogenesis (Takeichi, 1988; Nose et al., 1988; Miyatani et al., 1989).

In the present study we found that differentiated human carcinoma cell lines generally express E-cadherin and are noninvasive in vitro, whereas dedifferentiated cell lines do not express this cell–cell adhesion molecule and are invasive. Furthermore, we could correct the invasive behavior of dedifferentiated breast carcinoma cells by transfection with E-cadherin cDNA. These results indicate that E-cadherin expression is crucial for the maintenance of the differentiated noninvasive state of epithelial cells.

Materials and Methods

Cell Lines and Cell Culture

The various differentiated and dedifferentiated human carcinoma cell lines were obtained from the tumor bank of the German Cancer Research Center (DKFZ; Heidelberg, Germany) and the American Type Culture Collection (ATCC). The following cell lines were used: RT4 and RT112 (DKFZ; from bladder carcinomas, differentiated); EJ28 (DKFZ; from a bladder carcinoma, dedifferentiated); CX-1 (DKFZ), WiDr, HCT116, SW948, and COLO205 (ATCC; from colon carcinomas, differentiated); SW620 (ATCC; from a colon carcinoma, dedifferentiated); MCF-7 (DKFZ), and MDA-MB-361 (ATCC; from breast carcinomas, differentiated); BT-549 (ATCC; from a breast carcinoma, intermediate phenotype); MDA-MB-231, -435S, and -436 (DKFZ; from breast carcinomas, dedifferentiated); LX-1 (DKFZ; from a lung carcinoma, differentiated); A-427 and A-549 (DKFZ; from lung carcinomas, intermediate phenotypes); LXF289 and SK-MES-1 (DKFZ; from lung carcinomas, dedifferentiated); Capan-1, Capan-2, and DAN-G (DKFZ; from pancreas carcinomas, differentiated); Hs 766T (DKFZ; from a pancreas carcinoma, intermediate phenotype); MIA PaCa-2 (ATCC; from a pancreas carcinoma, dedifferentiated). For more information on these cell lines see Table I. A-431 human vulva carcinoma cells were obtained from ATCC, MRC-5 human lung fibroblasts were purchased from Flow Laboratories Inc. (McLean, VA), and CSG 120/7 mouse salivary gland carcinoma cells were from L. M. Franks, London (Knowles and Franks, 1977). Cells were grown in DME and 10% FCS under standard cell culture conditions. The invasion assay on collagen gels was performed as described (Behrens et al., 1989).

Purification of the 80-kD Tryptic Fragment of Human E-cadherin and Production of mAbs

Our rabbit anti-canine Arc-1 antiserum (Behrens et al., 1989), which recognizes the human homologue was used for affinity purification of the 80-kD tryptic fragment of E-cadherin from human A-431 carcinoma cells (for the expression of E-cadherin in A-431 cells see Damsky et al., 1983; Shimoyama et al., 1989*a*). Tryptic digests of the cells (Behrens et al., 1989) were chromatographed on an affinity column prepared by coupling 40 mg of the IgG fraction of the antiserum to 5 ml CNBr-Sepharose. The 80-kD fragment could then specifically be eluted with 5 mM EDTA, 500 mM NaCl, 50 mM Tris-Cl, pH 8.5, and concentrated by acetone precipitation at −70°C. From 1 g of wet cells 1.5 μg of 80-kD tryptic fragment of human E-cadherin could be isolated.

For the production of mAbs, BALB/c mice were immunized with the antigen (3 μg per animal and immunization) using the ABM adjuvans system (Zymed Laboratories Inc., South San Francisco, CA). Spleen cells from immunized mice were fused with P3-X63-Ag 8.653 mouse myeloma cells (Kearney et al., 1979). The resulting hybridomas were cultured in multiwell tissue culture plates.

Screening of mAbs

Hybridoma supernatants were screened in an enzyme-linked immunoassay using 96-well microtiter plates (Nunc, Roskilde, Denmark) which were coated with 75 ng/well of the tryptic fragment of human E-cadherin. Antibodies bound to the solid-phase antigen were detected using peroxidase-coupled goat anti-mouse IgG antibodies (Jackson Immuno Research Laboratories, Avondale, PA). Positive hybridoma supernatants were rescreened by immunofluorescence on human A-431 carcinoma cells and on frozen sections of human small intestine following fixation with 3% formaldehyde and treatment with 0.5% Triton X-100, and by Western blot analysis of the 80-kD fragment and of total A-431 cell extracts. For preparation of these extracts, freshly scraped A-431 cells were lysed for 5 min at 96°C with 2% SDS, 5% 2-mercaptoethanol in L-CAM assay buffer (Cunningham et al., 1984) followed by centrifugation at 100,000 *g* at 12°C (Behrens et al., 1989). Antibody isotypes were determined using a mouse monoclonal isotyping kit (Serotec, Oxford, England).

Analysis of E-cadherin in the Various Human Carcinoma Cell Lines using Immunological Methods

For the immunofluorescence of E-cadherin in tissue culture cells, 100,000 *g* hybridoma supernatants or Protein A–purified antibodies (eluted from Protein A-Sepharose with 50 mM ethanolamine, pH 11) were used. Immunofluorescence of cytokeratins was carried out according to Sun and Green (1978) using broad-spectrum antibodies (Jackson ImmunoResearch Laboratories and Camon Labor Service, Wiesbaden, Germany). For comparing cell lines in Western blot experiments, 50 μg total cell protein (extracted with SDS-buffer, see above) were loaded per gel lane. For quantitative antibody binding, human carcinoma cells were cultured overnight in 96-well flexible microtiter plates (5×10^4 cells/well), fixed with 3% formaldehyde, and permeabilized with Triton X-100. After 1-h incubation with

hybridoma supernatant (which was preabsorbed over fixed and permeabilized MRC-5 human lung fibroblasts for 1 h at room temperature) cells were washed and incubated with goat anti-mouse IgG (Jackson Immuno Research Laboratories Inc., Avondale, PA) labeled with ^{125}I using the lactoperoxidase method (2×10^7 cpm/μg; 5×10^5 cpm/well). Wells were then cut off and radioactivity of triplicate experiments was determined in a gamma counter.

Northern Blot Analysis

To obtain a human E-cadherin cDNA-probe, a human liver cDNA library (in the plasmid expression vector pUEX1; a gift of U. Loggen, Essen, Germany) was screened. Out of 1.6×10^5 clones, HC6-1 was picked and the insert was subcloned into M13 phages and sequenced in both directions by the dideoxy method using T7 DNA polymerase (Pharmacia Fine Chemicals, Piscataway, NJ).

For preparation of RNA, cells were washed with PBS, scraped off the plates with a rubber policeman, and lysed on ice in 1% NP40, 140 mM NaCl, 1.5 mM $MgCl_2$, 100 U RNasin/ml, 10 mM Tris-Cl, pH 8.0. After centrifugation at 12,000 rpm in a rotor (model JA20; Beckman Instruments, Inc., Palo Alto, CA), the supernatant was extracted at 65°C with acid phenol containing 1% SDS, 10 mM EDTA, followed by extraction at room temperature with Tris-buffered phenol, phenol/chloroform, chloroform, and precipitation with ethanol at −20°C. Poly A^+-RNA was obtained by affinity chromatography of total RNA on oligo (dT)-cellulose using the spun column method (Pharmacia Fine Chemicals). RNA was electrophoresed on glyoxal gels (Maniatis et al., 1982) followed by blotting on Hybond-N membranes (Amersham Corp., Arlington Heights, IL). Hybridization of the blots was carried out with nick-translated cDNA-probes overnight at 64°C in 5× SSC, 5× Denhardt's solution, 10% dextransulfate (Pharmacia Fine Chemicals), 0.5% SDS, 100 μg/ml heated salmon sperm DNA. Filters were washed at room temperature with 2× SSC (2 × 10 min) and then with 0.5× SSC, 0.1% SDS at 64°C (two times 15 min) followed by autoradiography with intensifying screens at −70°C.

Transfection of Mouse E-cadherin cDNA into Dedifferentiated Human Carcinoma Cells

A subclone of dedifferentiated human breast carcinoma cells (i.e., MDA-MB-435S/1) was transfected using the Ca^{2+}-phosphate coprecipitation method with the full-length cDNA of mouse E-cadherin (plasmid pBATEM 2; E-cadherin under the control of the chicken β-actin promoter; a gift of A. Nagafuchi and M. Takeichi, Kyoto, Japan) together with a plasmid for neomycin resistance. G 418-resistant clones which exhibited an epithelioid morphology were analyzed for mouse E-cadherin expression by immunofluorescence using supernatants of the DECMA-1 rat hybridoma (a gift of R. Kemler, Freiburg, Germany). Invasion of the transfected cells into collagen matrices was studied as described above.

Results

Characterization of Probes

Mouse mAbs against the 80-kD tryptic fragment of human E-cadherin were generated and characterized. Antibody 6F9 (an IgG1) reacted most specifically with the 120- and 80-kD E-cadherin bands on Western blots (Fig. 1, *a* and *b*; *c* and *d* show the reaction with a second antibody, 15C12, and *e* and *f* is the control reaction with the polyclonal rabbit anti-canine Arc-1 antibody, see also Behrens et al., 1989). In the immunofluorescence of frozen sections antibodies 6F9 and 15C12 specifically stained the epithelium of the human small intestine; staining was confined to the lateral borders of the epithelial cells, and some enrichment was seen in the area of the junctional complex (data not shown, cf. also Behrens et al., 1985).

A partial E-cadherin cDNA clone, HC6-1, was isolated from a human liver plasmid cDNA library, and the insert was sequenced. It encompasses 386 bp, which are homologous to position 865–1251 of the full-size mouse E-cadherin cDNA (Nagafuchi et al., 1987), and the first 142 bp are identical to the 3′ end of the published sequence of another partial human E-cadherin cDNA clone (Mansouri et al., 1988). The sequence of HC6-1 has the number X 52279 in the EMBL data bank.

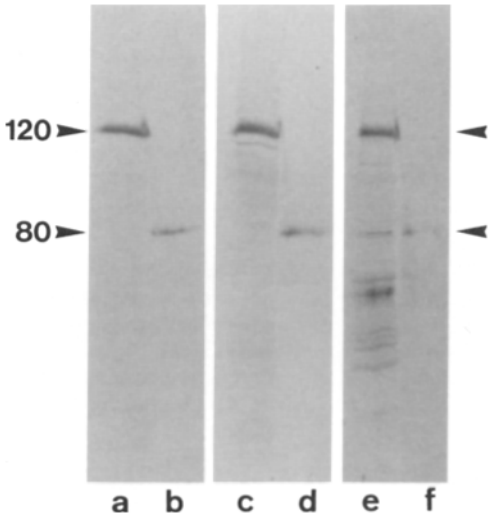

Figure 1. Western blot analysis of A-431 human carcinoma cell extracts stained for E-cadherin with the new mAbs. Total cell extracts (lanes *a*, *c*, and *e*) and tryptic surface digests (lanes *b*, *d*, and *f*) of A-431 human vulva carcinoma cells were analyzed for the presence of E-cadherin using the antibodies 6F9 (lanes *a* and *b*) and 15C12 (lanes *c* and *d*). As a control for the migration of the mature 120-kD protein and the tryptic 80-kD fragment, the reaction with our polyclonal rabbit anti-canine Arc-1 antiserum is also shown (lanes *e* and *f*). This antiserum was used for the isolation of the human 80-kD E-cadherin fragment which served as immunogen for the preparation and characterization of the new mAbs. (see Materials and Methods).

Expression of E-cadherin in Differentiated and Dedifferentiated Human Carcinoma Cell Lines

A variety of differentiated and dedifferentiated human carcinoma cell lines were obtained from the tumor cell bank of the German Cancer Research Center (DKFZ) and from the American Type Culture Collection (ATCC). We observed that cell lines isolated from well differentiated bladder, breast, lung, and pancreas carcinomas exhibited an epithelioid morphology in tissue culture, whereas most cell lines obtained from the respective poorly differentiated tumors expressed a fibroblastoid shape (for more characteristics of these cell lines see Table I). A few cell lines expressed an intermediate phenotype. The state of differentiation in the tumors and in the resulting cell lines did not always correspond, e.g., in the case of colon carcinomas. We confirmed by immunofluorescence of cytokeratins using a broad-spectrum antiserum that all cell lines were of epithelial origin (data not shown).

By applying the new mAbs and the human cDNA probe we found that the differentiated (epithelioid) carcinoma cell lines expressed E-cadherin, whereas the dedifferentiated (fibroblastoid) lines were usually negative. For instance, when tested by immunofluorescence with antibody 6F9, the differentiated bladder carcinoma cell line RT4 expressed E-cadherin in the areas of cell–cell contact (Fig. 2, *a* and *b*), whereas the dedifferentiated bladder carcinoma cell line

Table I. Characterization of the Human Carcinoma Cell Lines

Cell line		Characteristics related to differentiation	Morphology of cultured cells*
Bladder			
	RT4	derived from a well-differentiated carcinoma (Rigby and Franks, 1970)	E
	RT112	derived from a well-differentiated carcinoma (Steele et al., 1983)	E
	EJ28	derived from an anaplastic carcinoma (Hastings and Franks, 1983)	F
Colon			
	CX-1	well-differentiated cell line (Daneker et al., 1989)	E
	WiDr	derived from an adenocarcinoma, differentiated cell line (Noguchi et al., 1979)	E
	HCT116	epithelioid cell line in tissue culture (our finding; compare with Boyd et al., 1988)	E
	SW948	derived from an undifferentiated adenocarcinoma, differentiated in culture (Leibowitz et al., 1976)	E
	COLO205	derived from an anaplastic adenocarcinoma, differentiated in tissue culture (Semple et al., 1978)	E/Sph‡
	SW620	dedifferentiated cell line (Leibowitz et al., 1976)	Sph‡
Breast			
	MCF-7	characteristics of differentiated mammary epithelium; estrogen receptor-positive§ (Soule et al., 1973; Engel and Young, 1978)	E
	MDA-MB-361	estrogen-receptor-positive cell line (Engel and Young, 1978)	E
	BT-549	derived from an invasive ductal tumor (ATCC)	F/E‖
	MDA-MB-231	derived from a poorly differentiated carcinoma, spindle-shaped in culture; estrogen receptor-negative (Cailleau et al., 1974; Engel and Young, 1978)	F
	MDA-MB-435S	spindle-shaped cell line (Cailleau et al., 1978)	F
	MDA-MB-436	spindle-shaped cell line (Cailleau et al., 1978)	F
Lung			
	LX-1	derived from a human tumor which was characterized as a moderately well-differentiated adenocarcinoma after passage in nude mice (H. Löhrke, Heidelberg, personal communication)	E
	A-427	epithelial-like cell line, many cells with bizarre shapes (Giard et al., 1973)	E/F‖
	A-549	some characteristics of type II alveolar epithelial cells; vimentin-positive (Lieber et al., 1976; Blobel et al., 1984)	F/E‖
	LXF289	derived from a moderately well-differentiated adenocarcinoma; dedifferentiated morphology in culture (H. Löhrke, Heidelberg, personal communication)	F
	SK-MES-1	cell line from a squamous carcinoma; vimentin-positive (Fogh and Trempe, 1975; Blobel et al., 1984)	F
Pancreas			
	Capan-1	well-differentiated adenocarcinoma cells (Fogh et al., 1977)	E
	Capan-2	adenocarcinoma cell line (Fogh et al., 1977)	E
	DAN-G	derived from a well-differentiated carcinoma (H. Löhrke, Heidelberg, personal communication)	E
	Hs 766T	derived from a lymph node metastasis (Owens et al., 1976)	F/E‖
	MIA PaCa-2	derived from an undifferentiated carcinoma (Yunis et al., 1977)	F

* Cells were grouped according to their morphology in cell culture as epithelioid (*E*) or fibroblastoid (*F*).
‡ Sph, cells showed a spherical morphology.
§ The estrogen receptor is a widely used marker for differentiated mammary carcinomas (Engel and Young, 1978).
‖ Cell lines with intermediate morphology, whereby the first letter indicates the dominant form.

EJ28 did not show any fluorescence (Fig. 2, *c* and *d*). Similarly, the differentiated breast (MCF-7; Fig. 2, *e* and *f*), lung (LX-1; Fig. 3, *a* and *b*) and pancreas (DAN-G; Fig. 3, *e* and *f*) carcinoma cell lines expressed E-cadherin whereas the corresponding dedifferentiated breast (MDA-MB-435S; Fig. 2, *g* and *h*), lung (LXF289; Fig. 3, *c* and *d*), and pancreas (MIA PaCa-2; Fig. 3, *g* and *h*) carcinoma cell lines were negative.

The total amount of E-cadherin in these cell lines, as observed by Western blotting, largely corresponded with the immunofluorescence data. For instance, extracts of the differentiated bladder and breast carcinoma cell lines were positive for the 120-kD polypeptide, whereas the dedifferentiated bladder and breast carcinoma cell lines were negative (Fig. 4). These differences were also seen with the second antibody 15C12 (data not shown). Northern blots revealed similar differences in the E-cadherin mRNA content. For example, the differentiated bladder, breast, lung, and pancreas carcinoma cell lines expressed E-cadherin-mRNA well, whereas the corresponding dedifferentiated carcinoma cell lines did not show this message (Fig. 5). Cell lines with intermediate phenotypes (e.g., A-427 and Hs 766T) expressed small amounts of mRNA.

We also examined the human carcinoma cell lines (in par-

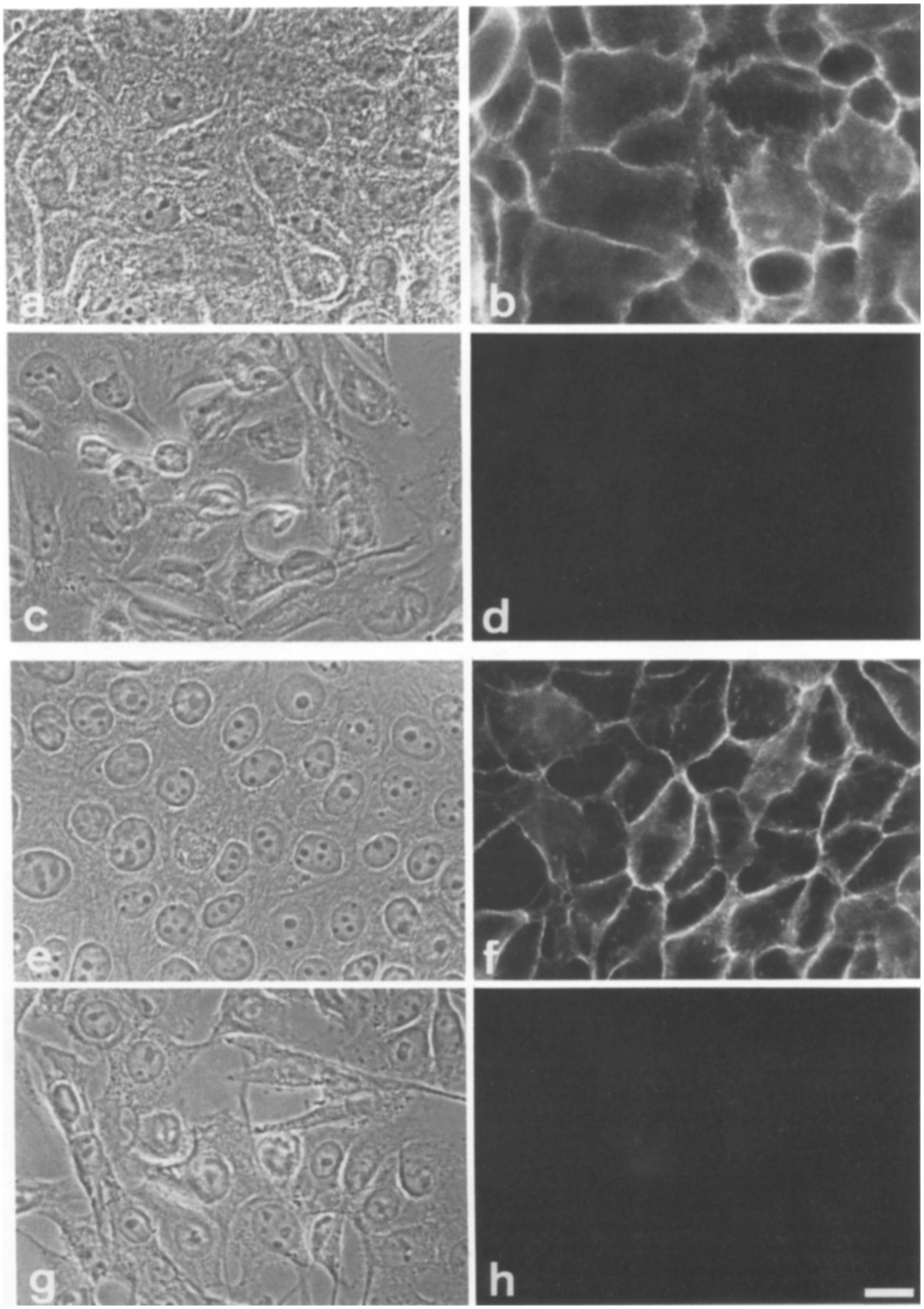

Figure 2. Immunofluorescence staining for E-cadherin in human bladder and breast carcinoma cell lines. The new mAb 6F9 was used in this experiment. (*a* and *b*) Differentiated bladder carcinoma cell line RT4; (*c* and *d*) dedifferentiated bladder carcinoma line EJ28; (*e* and *f*) differentiated breast carcinoma cell line MCF-7; and (*g* and *h*) dedifferentiated breast carcinoma line MDA-MB-435S. Apparently, the differentiated cell lines express E-cadherin at their cell–cell contact sites, whereas the dedifferentiated lines are negative. Bar, 20 μm.

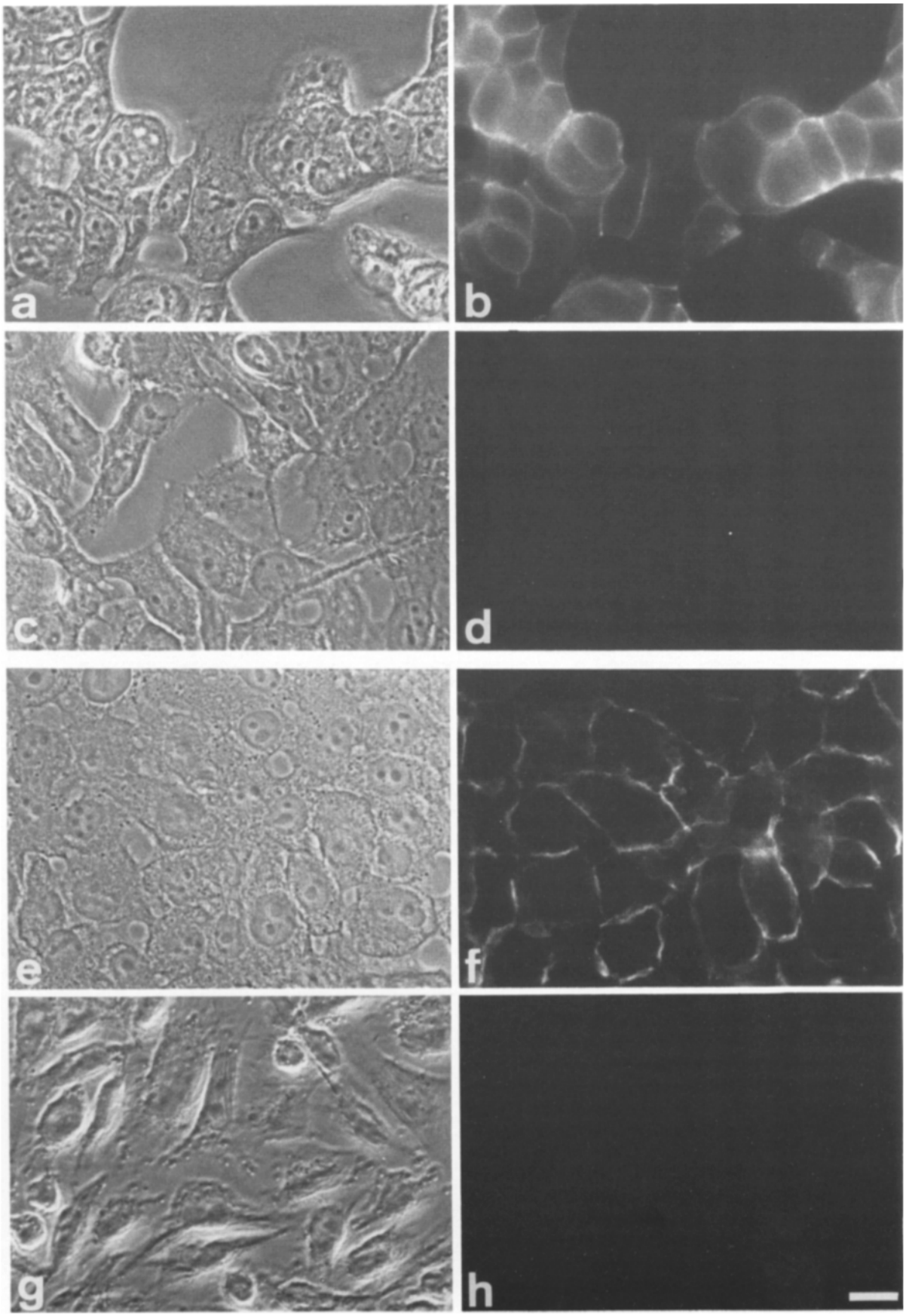

Figure 3. Immunofluorescence staining for E-cadherin in human lung and pancreas carcinoma cell lines. mAb 6F9 was used. (*a* and *b*) Differentiated lung carcinoma cell line LX-1; (*c* and *d*) dedifferentiated lung carcinoma line LXF289; (*e* and *f*) differentiated pancreas carcinoma cell line DAN-G; and (*g* and *h*) the dedifferentiated pancreas carcinoma cell line MIA PaCa-2. The differentiated lines express E-cadherin at the cell–cell contact sites, whereas the dedifferentiated lines are negative. Bar, 20 μm.

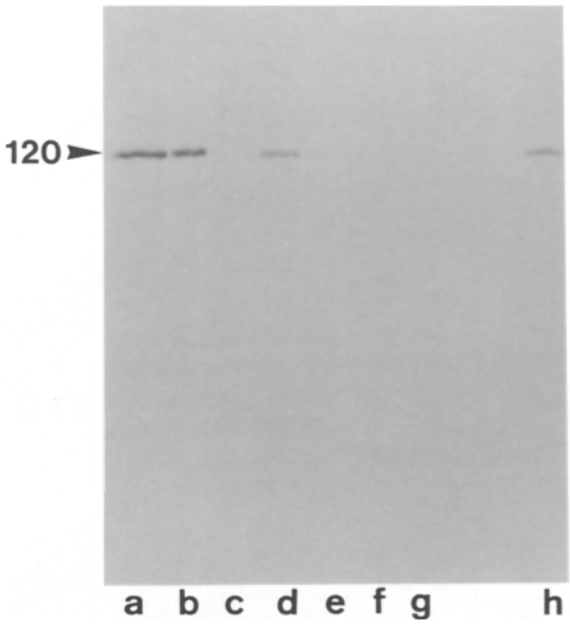

Figure 4. Western blot of total carcinoma cell extracts stained for E-cadherin. (lanes *a* and *b*) Extracts of the differentiated bladder carcinoma cell lines RT112 and RT4; (lane *c*) of the dedifferentiated bladder carcinoma line EJ28; (lane *d*) of the differentiated breast carcinoma cell line MCF-7; and (lanes *e* and *g*) of the three dedifferentiated breast carcinoma lines MDA-MB-231, -435S, and -436 were analyzed using mAb 6F9; (lane *h*) is a control extract from differentiated human A-431 vulva carcinoma cells. Apparently, the differentiated cell lines express E-cadherin whereas the dedifferentiated lines are negative.

ticular the ones which did not express E-cadherin) for the presence of intact E-cadherin genes by Southern blot analysis. When cellular DNA was cleaved with five different restriction enzymes (EcoRI, BamHI, HindIII, AccI, SacI) and hybridized with the HC6-1 probe, no significant differences in the migration and intensities of the fragments could be detected (data not shown). Apparently, the E-cadherin gene in the dedifferentiated carcinoma cell lines is not deleted, and we could not find any gross rearrangement in the region covered by our probe. We also tested whether the lack of expression of E-cadherin in dedifferentiated cell lines might be because of hypermethylation of the gene (cf., Feinberg and Vogelstein, 1983; Feinberg et al., 1988). In contrast, we found that the dedifferentiated cell lines rather exhibited a lower degree of methylation in the probed genomic region, as seen by new bands created by digestion with the enzyme HpaII (data not shown).

Correlation between E-cadherin Expression and Invasiveness In Vitro

We then examined the various human carcinoma cell lines for invasiveness in the collagen assay (Behrens et al., 1989) and found a striking inverse correlation with the degree of E-cadherin expression. Thus, E-cadherin positive cell lines were largely noninvasive for collagen gels, whereas the E-cadherin-negative lines did penetrate into the gel (Fig. 6). Clear-cut examples are the bladder carcinoma cell lines RT112, RT4, and EJ28; in the cases of the breast, lung, and pancreas carcinoma cell lines, a gradual shift from the E-cadherin-expressing noninvasive state to the nonexpressing invasive state was observed. The differentiated colon carcinoma cell lines were all noninvasive; the fact that a largely dedifferentiated cell line (SW620) did not invade might be because of the predominantly spherical morphology of the cells (see also Table I).

Prevention of the Invasiveness of Human Breast Carcinoma Cells by Transfection with E-cadherin cDNA

Mouse E-cadherin cDNA was transfected into human breast carcinoma cells MDA-MB-435S/1 (see Materials and Methods), and clones expressing various amounts of mouse E-cadherin were observed. Of these, three stable epithelioid clones were finally established: clone cad-B1 expressed mouse E-cadherin well at the cell–cell contacts, as shown by staining with the mAb DECMA-1 (Fig. 7, *a* and *b*), whereas a control clone neo-B4 did not (Fig. 7, *c* and *d*). Cad-B1-cells were still negative for human E-cadherin, as tested with antibody 6F9 (Fig. 7 *e*; note that the two antibodies DECMA-1 and 6F9 react in a species-specific manner with the mouse and human molecule, respectively; compare Fig. 7, *b*, *e*, and *f*).

The transfected breast carcinoma cells were then examined for invasiveness in vitro. The E-cadherin–expressing clone cad-B1 was found to be five times less invasive for collagen gels than the control clone neo-B4 (compare the black columns in Fig. 8, *a* and *b*) or the parental cell line (Fig. 6). However, full invasiveness of the cad-B1 cells could be regenerated by dissociating them with antibody DECMA-1 (which specifically recognizes mouse E-cadherin, compare the checkered columns in Fig. 8, *a* and *b*). As a control, DECMA-1 also induced invasiveness of mouse carcinoma cells CSG 120/7 (Fig. 8 *c*). These data indicate that E-cadherin expression converts human breast carcinoma cells MDA-MB-435S from a dedifferentiated invasive to a differentiated noninvasive state. They thus complement the experiments in which dedifferentiated invasive epithelial cells were generated by disturbance of E-cadherin–mediated cell–cell adhesion with specific antibody treatment (Fig. 8 *c*; see also Behrens et al., 1989).

Discussion

In this study we examined the expression of the cell–cell adhesion molecule E-cadherin (which has also been named Arc-1, uvomorulin, and cell-CAM-120/80) in a variety of human carcinoma cell lines originating from different tissues. Particular emphasis was directed toward changes of E-cadherin expression which occur in the process of tumor dedifferentiation. We found that cell lines derived from well differentiated human carcinomas (which keep the epithelial phenotype in tissue culture, 14 cell lines were tested) generally express E-cadherin well, whereas the molecule is not detected in most cell lines from poorly differentiated carcinomas (which exhibit a fibroblastoid phenotype in tissue culture, nine cell lines were tested). As a correlate, E-cadherin-negative cell lines were found to be invasive for collagen gels, whereas E-cadherin-positive lines did generally not enter the extracellular matrix. Furthermore, the invasiveness of dedifferentiated breast carcinoma cells could be corrected by transfection with E-cadherin cDNA. These results show

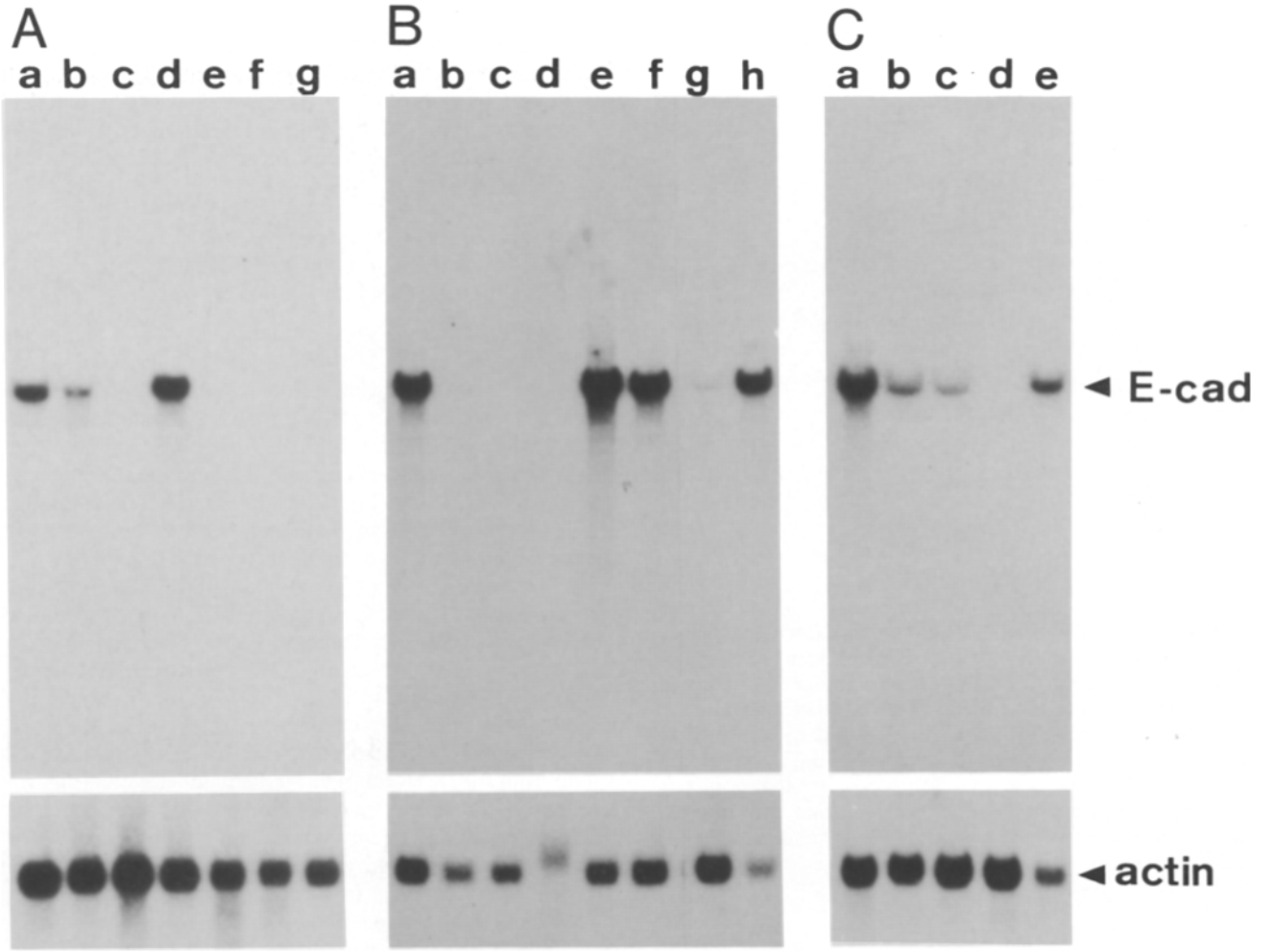

Figure 5. Northern blot analysis of various human carcinoma cell lines for the presence of E-cadherin mRNA. Electrophoresed poly A^+-RNA was blotted onto Hybond filters and hybridized with the HC6-1 cDNA probe (which recognizes E-cadherin mRNA) and with an actin cDNA probe (for the demonstration of the loading in the three experiments). (*A*) Bladder (lanes *a–c*) and breast (lanes *d–g*) carcinoma cell lines: (lane *a*) RT112; (lane *b*) RT4; (lane *c*) EJ28; (lane *d*) MCF-7; (lane *e*) MDA-MB-231; (lane *f*) MDA-MB-435S; and (lane *g*) MDA-MB-436. (*B*) Lung (lanes *a–d*) and pancreas (lanes *e–g*) carcinoma cell lines: (lane *a*) LX-1; (lane *b*) A-427; (lane *c*) SK-MES-1; (lane *d*) LXF289; (lane *e*) Capan-2 (lane *f*) DAN-G; and (lane *g*) Hs 766T. (lane *h*) Control mRNA from the bladder carcinoma cell line RT112. (*C*) Colon carcinoma cell lines: (lane *a*) CX-1; (lane *b*) HCT116; (lane *c*) SW620; (lane *d*) shows the absence of E-cadherin mRNA in MRC-5 human fibroblasts; (lane *e*) is control mRNA from the bladder carcinoma cell line RT112. Apparently, the differentiated carcinoma cell lines express E-cadherin mRNA, whereas the dedifferentiated lines are negative. The intermediate lines (A-427 and Hs 766T) express a small amount of mRNA (cf. with Table I).

that E-cadherin expression is a decisive indicator for differentiation and invasiveness of human carcinoma cells, and they suggest that E-cadherin should now thoroughly be examined for its possible role in preventing tumor cell invasion in vivo.

Specificity of Probes

We have here used new mAbs and a cDNA-probe for examining E-cadherin expression in the various human carcinoma cell lines. Since there exists a family of Ca^{2+}-dependent cell–adhesion molecules, how can we be sure that our probes are specific for the epithelial form of the cadherins? First, we do not need to consider N-cadherin here, since this molecule exhibits a clearly different protease digestion pattern and a grossly different tissue distribution (Hatta et al., 1985; Volk and Geiger, 1986; Takeichi, 1988). Second, the antigen used for immunization was prepared by affinity chromatography on a polyclonal anti–E-cadherin antibody (i.e., our anti-Arc-1), and antibodies seem not to crossreact between E- and P-cadherin (Behrens et al., 1985, 1989; Hatta et al., 1985; Takeichi, 1988; Shimoyama et al., 1989*a*). Third, our mAbs stain the epithelium of the human small intestine and the cell contacts of MCF-7 human breast carcinoma cells, which exclusively express E- but not P-cadherin (Shimoyama et al., 1989*a*). The sequence of the human cDNA-probe was found to be most closely related to mouse E-cadherin and it is identical in its overlapping region to a partial human E-cadherin cDNA (Nagafuchi et al.,

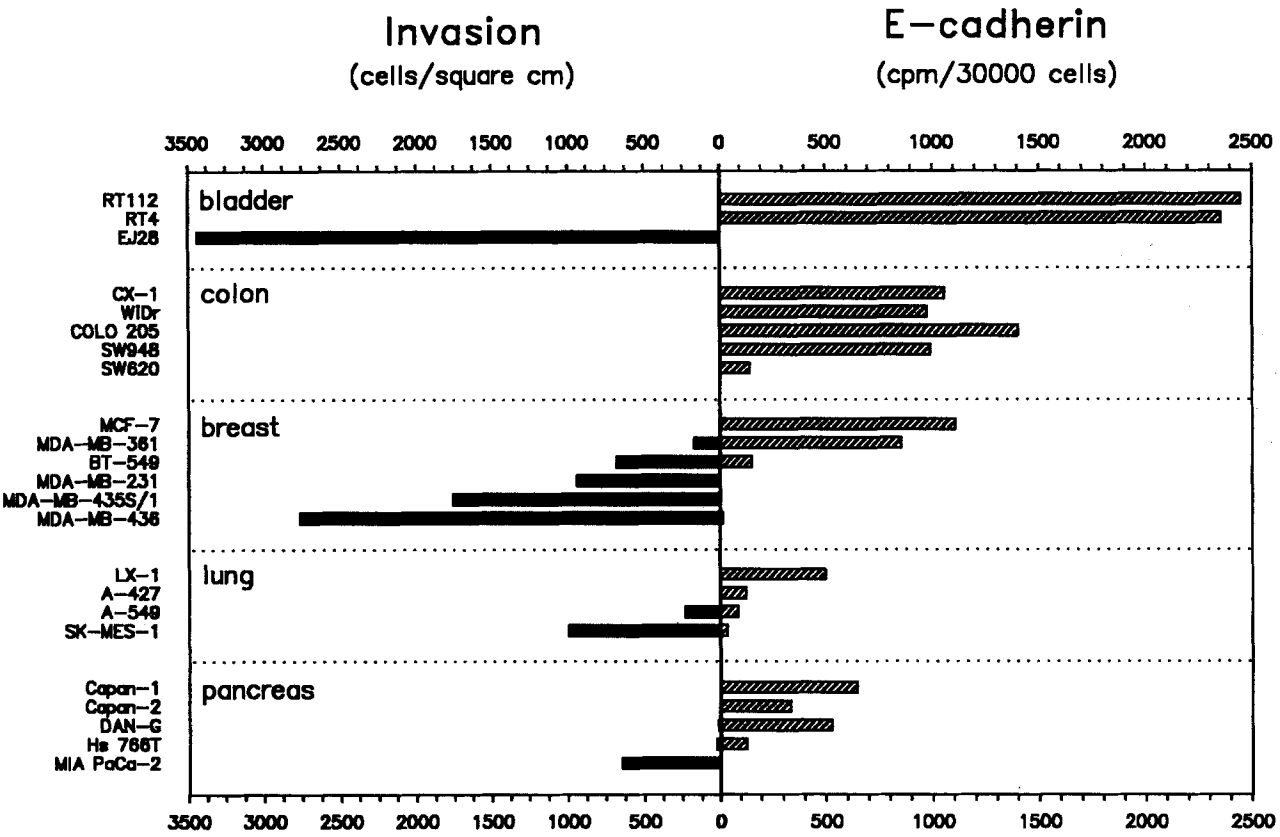

Figure 6. E-cadherin expression and invasiveness in vitro of the human carcinoma cell lines. For the invasion experiments, human carcinoma cell lines were cultured on collagen gels for 3 d and invasive cells were quantified in the light microscope (Behrens et al., 1989). The expression of E-cadherin was measured separately in an indirect cell binding assay using mAb 6F9 and ^{125}I-labeled goat anti-mouse IgG. The values were corrected for unspecific binding of an unrelevant mAb. Note the lack of invasion in the E-cadherin-expressing carcinoma cell lines and the invasive activity of the negative lines. The dedifferentiated lung carcinoma cell line LXF289 was not included in this graph, since it did not attach well to the microtiter dishes and therefore, binding could not be quantified. LXF289 was clearly E-cadherin negative in the other assays and did invade the collagen gel (322 cells/cm^2). The differentiated human vulva carcinoma cell line A-431 was noninvasive.

1987; Mansouri et al., 1988). Thus, both our antibodies and the cDNA-probe are specific and therefore suited for our purpose.

E-cadherin Is a Valuable Marker of Differentiated Noninvasive Human Carcinoma Cells

Previously, human MCF-7 breast carcinoma, human A-431 epidermoid carcinoma, and human JAR gestational choriocarcinoma cells (which are all of the differentiated type) were found to express E-cadherin (Damsky et al., 1983). No dedifferentiated carcinoma cell lines were examined in this early report. Recently, E-cadherin expression was studied in low and high metastatic variants of the murine ovarian carcinoma cell line OV2944. In analogy to our present findings, a highly metastatic subline expressed only small amounts, as compared with the high level found in weakly metastatic lines (Hashimoto et al., 1989). The cell lines we have used in the present study are of much greater variety of tissue origin, and they were particularly selected to belong to either the differentiated or dedifferentiated type. For instance, the bladder carcinoma cell lines RT112, RT4 (both differentiated), and EJ28 (dedifferentiated) were previously characterized for other epithelial markers; their cytokeratin expression pattern shows a good correlation with that of corresponding urothelial (transitional cell) carcinomas of the respective grade of malignancy (Moll et al., 1988). Furthermore, EJ28 cells metastasized from the bladder of nude mice whereas RT4 cells did not (Ahlering et al., 1987). These bladder carcinoma cell lines revealed a striking difference with respect to E-cadherin expression and corresponding invasiveness (Fig. 6). The breast carcinoma cell lines used were previously characterized as either hormone dependent (e.g., the differentiated line MCF-7, which expresses the estrogen-receptor) or hormone independent (three dedifferentiated MDA-MB-lines, which do not express the estrogen receptor; Engel and Young, 1978). In addition, the MDA-MB-lines 231, 435S, and 436 synthesize vimentin and only low levels of keratins (Sommers et al., 1989), which is also consistent with their dedifferentiated state. Our study now shows that E-cadherin represents an additional differentiation marker of breast carcinoma cell lines which inversely correlates with invasiveness in vitro. In conclusion, E-cadherin thus functions as a differentiation marker for a variety of human carcinoma cells, i.e., from bladder, breast, lung, pancreas, and possibly other carcinomas, which is of more generality than other markers mentioned above.

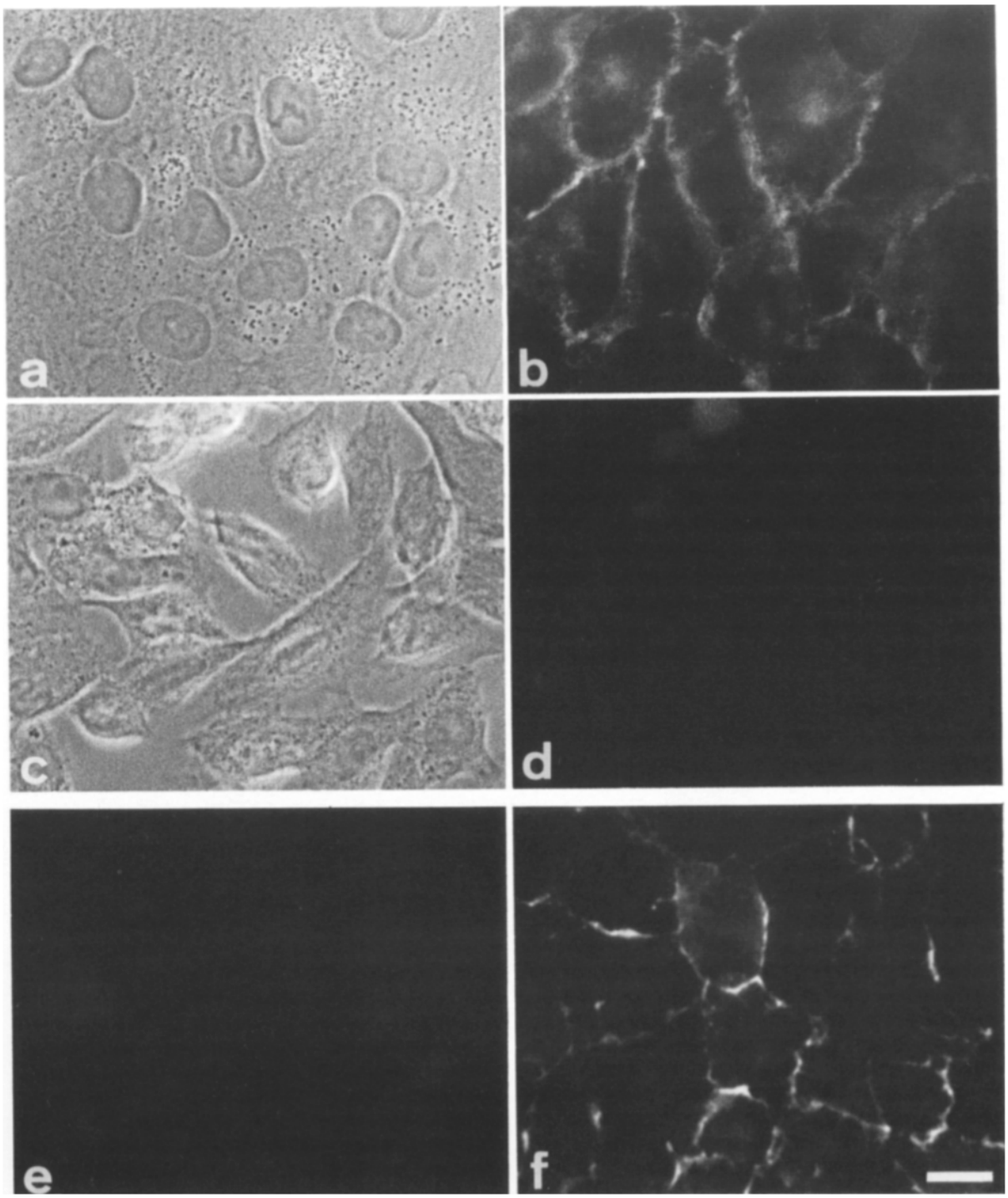

Figure 7. Immunofluorescence staining of mouse E-cadherin in transfected human breast carcinoma cells MDA-MB-435S/1. (*a*, *b*, and *e*) Human breast carcinoma clone cad-B1 transfected with mouse E-cadherin cDNA; (*c* and *d*) human control clone neo-B4; and (*f*) mouse CSG 120/7 carcinoma cells. The cells were stained with either mAb DECMA-1, which recognizes mouse E-cadherin (*b*, *d*, and *f*), or with antibody 6F9, which marks human E-cadherin (*e*). Note that the transfected clone cad-B1 expresses mouse E-cadherin at the cell–cell contacts. Bar, 20 μm.

E-cadherin expression of various human carcinomas was studied by others on tissue sections, and the molecule was found to be expressed in both differentiated and dedifferentiated tumors (Eidelman et al., 1989; Shimoyama et al., 1989*a*). However, much lower levels seem to be present in poorly differentiated carcinomas (see Fig. 7 *F* in Shimoyama et al., 1989*a*). We have recently also examined the expression of E-cadherin on sections of various human carcinomas. We found a good correlation with the state of differentiation in the cases of ovarian carcinomas (Pfisterer et al., 1990), of lobular breast carcinomas (Frixen, U. H., R. Moll, and W. Birchmeier, manuscript in preparation), and of squamous

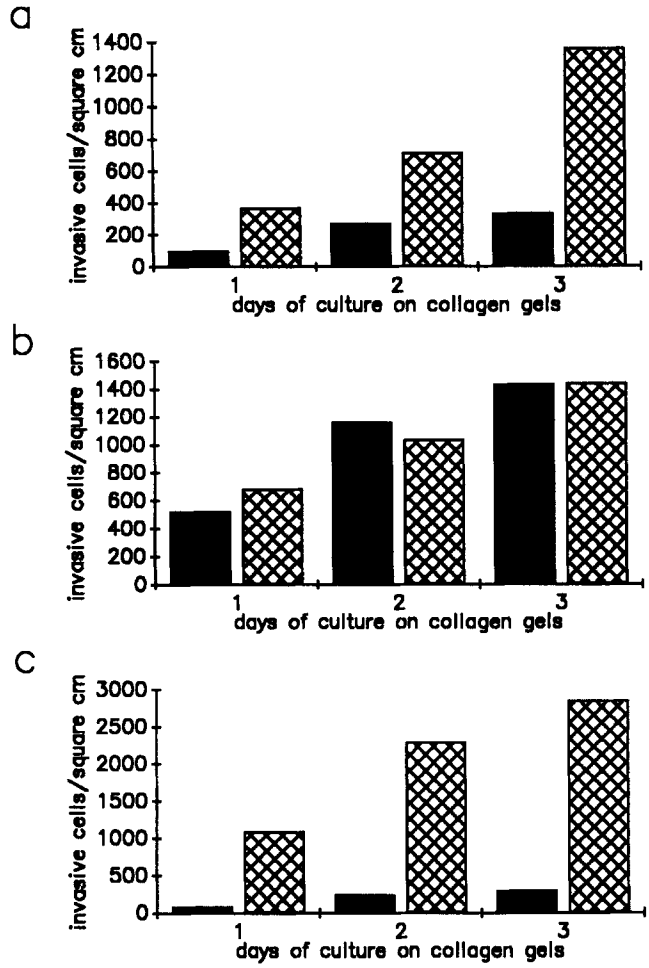

Figure 8. Invasiveness of human breast carcinoma cells MDA-MB-435S/1 transfected with mouse E-cadherin. Cells were plated on collagen gels and invasion was scored over 3 d as described in Materials and Methods. (*a*) E-cadherin expressing human breast carcinoma clone cad-B1; (*b*) negative human clone neo-B4; and (*c*) mouse CSG 120/7 carcinoma cells. Black columns, invasion in the absence of antibody; checkered columns, invasion in the presence of the dissociating anti-mouse E-cadherin mAb DECMA-1.

carcinomas of the head and neck (Schipper, J., U. H. Frixen, K. Jahnke, and W. Birchmeier, manuscript in preparation). An exception to the rule are ductal breast carcinomas; here invasive forms largely retain epithelial characteristics and express E-cadherin.

E-cadherin Triggers the Conversion between the Invasive and Noninvasive States of Carcinoma Cells

Considerable effort has been made in our laboratory and in the one of F. Van Roy and M. Mareel, Ghent, Belgium, to prove experimentally that E-cadherin expression is actually causal for inducing differentiation and preventing invasiveness of epithelial cells. First, transfection with E-cadherin cDNA converted both dedifferentiated breast carcinoma and *ras*-transformed MDCK cells toward the noninvasive phenotype (this report; Mareel et al., 1990). Second, treatment of differentiated salivary gland carcinoma and MDCK epithelial cells with anti-E-cadherin antibodies induced invasiveness (Fig. 8 *c*; Behrens et al., 1989). These combined data suggest that E-cadherin represents a kind of master molecule for regulating the maintenance of epithelial differentiation. However, other factors can also come into play; for instance, scatter factor induces invasiveness of MDCK epithelial cells without affecting E-cadherin expression (Weidner et al., 1990), suggesting that this factor acts via another mechanism.

Control of E-cadherin Gene Expression

We have observed that the relative amounts of E-cadherin protein and mRNA largely correspond to each other in the various carcinoma cell lines tested. When the E-cadherin gene was examined by Southern blotting, no deletion or gross rearrangement was yet detected in the nonexpressing cell lines. Thus, these results suggest that E-cadherin expression is largely controlled on the transcriptional level. To further examine the molecular mechanisms that regulate E-cadherin gene expression, the putative promoter of the E-cadherin gene has recently been isolated and characterized. A 270-bp genomic fragment (between −178 and −92) was found to be sufficient to induce a 20-fold higher CAT (chloramphenicol acetyltransferase) expression in epithelial cells than in fibroblasts, indicating that *cis*-regulatory elements control epithelial-specific E-cadherin expression (Behrens, J., and W. Birchmeier. 1990). *J. Cell Biol.* 111:157).

In human hepatocellular carcinomas a loss of heterozygosity on chromosome 16 was recently detected in 52% of the informative cases, and the common region of allele loss was localized between the positions 16 q 22.1 and 23.2 (Tsuda et al., 1990). The human E-cadherin gene is located at position 16 q 22.1 (Natt et al., 1989). Furthermore, loss of heterozygosity on chromosome 16 was much more frequent in poorly differentiated than in well-differentiated liver carcinomas. Thus, the E-cadherin gene is a good candidate for the tumor suppressor gene of chromosome 16 in hepatocellular carcinomas. Accordingly, the tumor suppressor gene "fat" of Drosophila has recently been identified as a cadherin-like molecule (C. Goodman, personal communication).

Comparison with Other Components Involved in Invasion and Metastasis of Human Carcinomas

Recently, the nm 23/awd gene product was identified by Steeg and collaborators, which is reduced in human melanomas, carcinomas, and sarcomas of high metastatic potential, and which is homologous to the developmentally regulated gene abnormal wing discs of Drosophila (Rosengard et al., 1989). Reintroduction of this gene (which encodes for a cytoplasmic protein) into highly metastatic cells led to a correction of the metastatic phenotype. The DCC gene, which is located on human chromosome 18 q, was found to be affected in 70% of colorectal tumors (Fearon et al., 1990). This gene codes for a putative cell-adhesion molecule of the N-CAM type, i.e., belongs to the immunoglobulin superfamily. The tumor marker CEA, which also belongs to the immunoglobulin superfamily, was found to function as a cell–cell adhesion molecule in colon adenocarcinoma cells (Benchimol et al., 1989). It is therefore likely that both cytoplasmic and cell surface proteins influence invasion and metastasis. The components discussed above (nm 23, DCC, 16q 22.1 - 23.2, E-cadherin, fat) seem to promote the pro-

cess when lost, i.e., might represent true invasion and metastasis suppressors.

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