Ela Induces the Expression of Epithelial Characteristics

Steven M. Frisch
La Jolla Cancer Research Foundation, La Jolla, California 92037

Abstract. Cells closely resembling epithelia constitute the first specific cell type in a mammalian embryo. Many other cell types emerge via epithelial–mesenchymal differentiation. The transcription factors and signal transduction pathways involved in this differentiation are being elucidated. I have previously reported (Frisch, 1991) that adenovirus Ela is a tumor suppressor gene in certain human cell lines. In the present report, I demonstrate that Ela expression caused diverse human tumor cells (rhabdomyosarcoma, fibrosarcoma, melanoma, osteosarcoma) and fibroblasts to assume at least two of the following epithelial characteristics: (a) epithelioid morphology; (b) epithelial-type intercellular adhesion proteins localized to newly formed junctional complexes; (c) keratin-containing intermediate filaments; and (d) down-regulation of non-epithelial genes. Ela thus appeared to partially convert diverse human tumor cells into an epithelial phenotype. This provides a new system for molecular analysis of epithelial-mesenchymal interconversions. This effect may also contribute to Ela's tumor suppression activity, possibly through sensitization to anoikis (Frisch, S. M., and H. Francis, 1994. J. Cell Biol. 124:619–626).

The Ela gene of adenovirus has been extensively investigated for diverse reasons. When introduced into various human tumor cell lines, it acts as a tumor suppressor gene, not by killing tumor cells, but by converting them into a nontransformed phenotype (Frisch, 1991). It regulates the transcription of several cellular genes positively or negatively (Shenk and Flint, 1991; Flint and Shenk, 1990; Jones et al., 1988; Braithwaite et al., 1991) and represses a large number of promoters in transient transfection assays (Rochette-Egly et al., 1990). It facilitates the transformation of primary rodent cells by oncogenes such as ras (Ruley, 1983). It blocks the differentiation in culture of pheochromocytoma cells into neurons (Maruyama et al., 1987) or myoblasts into myotubes (Webster et al., 1988). In addition, Ela complexes with proteins such as retinoblastoma protein, p107 and p130, thereby interfacing with the cell cycle machinery via cyclins and the transcription factor E2F (reviewed in Moran, 1993; Dyson and Harlow, 1992).

The epithelial cell is characterized by its morphology, extensive intercellular interactions through cell adhesion molecules (Ekblom et al., 1986; Grunwald, 1991; Schmidt et al., 1993), cell polarity (Rodriguez-Boulan and Nelson, 1992), keratin-containing intermediate filaments (Steinert and Roop, 1988), and synthesis of basement membrane proteins. The epithelial-specific intercellular adhesions consist of adherens junctions, tight junctions, and desmosomes. The major proteins involved forming these structures are: cadherins and catenins (adherens junctions; reviewed in Takeichi, 1991; Geiger and Ayalon, 1992), ZO-1, cingulin, and occludin (tight junctions; reviewed in Citi, 1993), desmoplakin, desmoglein, desmocollin, and plakoglobin (desmosomes; reviewed in Koch et al., 1991; Buxton et al., 1993).

These adhesions are critical for forming selectively permeable epithelial sheets, for establishing cellular polarity and for regulating pattern formation in epithelial tissues (reviewed in Schmidt et al., 1993; Birchmeier and Birchmeier, 1993; Grunwald, 1991).

The results reported here show that the expression of Ela in certain nonepithelial cells induced the expression of genes encoding various epithelial cell-specific adhesion proteins and the formation of the appropriate intercellular adhesions. Other epithelial features, such as synthesis of keratin-containing intermediate filaments, were induced in some of the cell lines as well. As such, Ela appeared to be capable of reprogramming gene expression to produce a partial or complete epithelial phenotype, depending upon the original cell type.

Materials and Methods

Cell Lines

The tumor cell lines used in this study, A204 rhabdomyosarcoma, HT1080 fibrosarcoma, A2058 melanoma, and Saos-2 osteosarcoma and RD rhabdomyosarcoma were obtained from American Type Culture Collection (Rockville, MD) and subcloned before use. MCF-7 cells were from American Type Culture Collection and were used without subcloning. The Li-Fraumeni fibroblast cell line 172 (Bischoff et al., 1990) was obtained from Dr. Michael Tainsky (M. D. Anderson Cancer Center, Houston, TX). It had been passaged until loss of the wild-type allele of p53 was achieved (M. Tainsky, personal communication). HaCat cells were from R. Fusenig (German Cancer Center, Heidelberg, Germany). Construction of Ela derivatives of the H4 subclone of HT1080 (Frisch and Francis, 1994) and of A2058 melanoma cells (Frisch, 1991) were described. The Ela derivatives of the other cell lines were constructed using methods described previously (Frisch and Francis, 1994) with the retrovirus that contained the 12 S form of adenovirus-5 Ela under control of the SV-40 early promoter.
Western Blotting

Western blots were performed using Immobilon filters (Millipore Corp., Bedford, MA), horseradish peroxidase-conjugated secondary antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN) and ECL detection (Amersham Corp., Arlington Heights, IL). Primary antibodies were from the following sources: anti-Ela clone M73 (Oncogene Science Inc., Manhasset, NY), anti-desmoplakin polyclonal antibody pN6 (Thea Lea Green, Northwestern University, Evanston, IL), or anti-desmoglein monoclonal antibody (Pamela Cowin, New York University, New York), anti-desmoglein monoclonal antibody clone 3.10 (BioDesign, Kennebunkport, Maine), anti-ZO-1 polyclonal antibody no. 7445 (James Anderson, Yale University, New Haven, CT), anti-pan-cadherin polyclonal antibody C3678 (Sigma Chemical Co., St. Louis, MO), anti-E-cadherin (DECMA-1; Sigma), anti-desmin (Oncogene Science; clone DE-B1). In all Western blots, equal protein loading of the SDS-PAGE gels was confirmed by Amido black staining of the blots after development with the ECL reagent.

Northern Blotting

Northern blotting was performed using formaldehyde-agarose gels and nitrocellulose (Schleicher & Schuell, Keene, NH) filters on RNAs (12 μg) that were prepared by guanidinium thiocyanate/cesium chloride gradients. Equal RNA loading was verified by photographing the ethidium bromide-stained gels prior to transfer. cDNA probes were made random-primer labeling (Stratagene Corp., La Jolla, CA) of restriction fragments in low-melt agarose gel slices. Probes were from the following sources: human desmoplakin (pDPI/II; K. Green, Northwestern University, Evanston, IL), 1,800-bp HindIII fragment), human desmoglein-2 (W. Franke, German Cancer Research Institute, Heidelberg; phdsg2.pca, 800-bp EcoRI fragment), human plakoglobin (W. Franke, pHPGCd2.1, 1540 bp SacI fragment), E-cadherin (pCOS5; David Rimm, Yale University), human keratin 18 (pK189; R. Oshima, La Jolla Cancer Research Foundation; EcoRI/BamHI 1,076-bp fragment), human fibronectin (pBS70K; Alex Morla, La Jolla Cancer Research Foundation; EcoRI/Xbal 592-bp fragment), glyceraldehyde-phosphate-dehydrogenase (pGAPDH; ATCC, 800-bp Xbal/PstI fragment), tyrosinase (pKS-E1-3.4-HR2.1; M. Spritz, Yale University; 2.1-kb HindIII-EcoRI fragment).

Immunofluorescence

Cells were grown on 2 cm gelatin-coated coverslips. Fixation was either with cold methanol (1 min for desmoplakin or desmoglein, 10 min for keratins) or with 1% paraformaldehyde for other proteins (Koch et al., 1991; Buxton et al., 1993). Cells (2 × 10^5) were plated onto Falcon filter inserts (25 mm) that had been precoated for 1 h with 100 μg/ml Matrigel (Collaborative Research, Lexington, MA). 2 d later, electrical resistance across the filters was measured using an epithelial voltohmeter (World Precision Instruments, Sarasota, FL).

Results

Ela Expression Causes Conversion to an Epithelioid Morphology

A retrovirus that transduces the 12 S (243-amino acids [aa]) form of adenovirus-5 Ela (Frisch, 1994) was used to generate stable Ela-expressing cell lines from the following parental cells: HT1080 fibrosarcoma (FS), A204 rhabdomyosarcoma (RM), RD rhabdomyosarcoma (RM2), Saos-2 osteosarcoma (OS), and fibroblasts spontaneously immortalized by deletion of p53 (Li-Fraumeni fibroblasts; FB); Ela derivatives of A2058 constructed by transfection were described (Frisch, 1991). Expression of Ela protein in G418-selected clones was verified by Western blotting (Fig. 1 a). All of the Ela-expressing cell lines adopted an epithelioid morphology (Fig. 1 b). Because of the large variation in Ela expression levels amongst different G418-resistant clones, individual clones were analyzed instead of pooling together large numbers of clones, which could have introduced the risk of unpredictable selection for a particular Ela expression level.

Expression of Epithelial-type Cell Adhesions of Ela-expressing Cell Lines

The morphologic properties of the Ela-expressing cell lines suggested that they had assembled epithelial-type junctional complexes. This was investigated by electron microscopy, immunofluorescence, Western blotting, and Northern blotting.

Desmosome formation requires the expression and assembly of desmosplakin, desmoglein, desmocollin, plakoglobin and other proteins (Koch et al., 1991; Buxton et al., 1993). Ela-expressing cell lines were subjected to immunofluorescence using anti-desmosplakin and desmoglein antibodies. Punctate pericytoplasmic structures closely resembling normal desmosomes were detected with desmoplakin antibody in Ela-expressing fibrosarcoma and rhabdomyosarcoma (Fig. 2) but neither in parental nor in Ela-expressing derivatives of other parental cell lines. In the fibrosarcoma cells, some perinuclear staining was also observed, which might reflect the binding of desmoplakin to intermediate filaments (Green and Jones, 1990). Although the desmosome density was lower in the Ela/FS than in the Ela/RM cells, well-formed desmosomes of double-leaflet structure (such as those arrowed) were readily detectable.

To determine whether Ela induced the expression of genes encoding desmosomal proteins, Northern blots were performed. The desmoplakin gene was induced by Ela in various extents in all of the tumor cell lines examined (although very weakly in the osteosarcoma cells) as well as in fibroblasts (Fig. 3). Expression of desmoglein-2 (DSG2), which is the form ubiquitously expressed in desmosome-bearing cells (Koch et al., 1991; Buxton et al., 1993), was induced by Ela in both of the rhabdomyosarcoma, melanoma, and...
Figure 1. Expression of Ela and its effect on cell morphology. (a) Expression of Ela protein in human tumor cells and fibroblasts infected with Ela retrovirus. Tumor cells were infected with the Ela-containing retrovirus, 4LNSX/8 (described in Frisch and Francis, 1994) or by transfection in the case of the ML cells (Frisch, 1991). After selection of G418-resistant clones, Ela expression was tested by Western blotting using anti-Ela antibody M73. Cell lines are abbreviated throughout as follows: fibroblasts (FB), osteosarcoma (OS), rhabdomyosarcoma (RM or RM2), melanoma (ML), or fibrosarcoma (FS). (b) Morphologies of representative Ela-expressing clones and "empty vector-infected" (control) cell lines were documented by phase-contrast microscopy.

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Figure 2. Assembly of desmosomes in Ela-expressing RM and FS cell lines, (Ela/RM or Ela/FS), or control cell lines (vec/RM, vec/FS). These were detected by immunofluorescence using anti-desmoplakin (α-DP) or anti-desmoglein (α-DG) antibodies, as described in Materials and Methods. Particularly well-formed desmosomes in the Ela/FS cells are arrowed.
Figure 3. Expression of desmosomal proteins in Ela-expressing cell lines. RNAs from vector-infected (Ela−) or Ela retrovirus-infected (Ela+) cell lines were probed on Northern blots (a) for desmoplakin (DP), desmoglein-2 (DSG2), plakoglobin (PG) or desmocollin-3 (DSC3) expression. To confirm expression of the corresponding protein of the correct molecular weight, Western blots (b) of indicated protein lysates were probed with DP or DSG2 antibodies, as described in Materials and Methods. Molecular weights were calculated relative to rainbow markers (Amersham Corp.). Protein from a human keratinocyte line HaCat was also run as a control.

Figure 4. Electron microscopic analysis of cell–cell adhesions in parental tumor cells (vec/FS, vec/RM) and Ela-expressing (Ela/FS, Ela/RM) derivative cells. Electron microscopy was carried out as described in Materials and Methods. The positions of electron-dense putative adhesional structures are arrowed. Bars, 1 μm.
fluent monolayers of RM cells with anthroyl-ouabain (Fig. 6). While the parental cells showed diffuse cytoplasmic distribution, the Ela/RM cells showed the lateral staining pattern that is characteristic of epithelial cells.

Functionality of the tight junctions was also tested by measuring the electrical resistance across confluent sheets of Ela-expressing cells, which were grown on matrigel-coated filter inserts. The empty vector-infected rhabdomyosarcoma cells had no measurable electrical resistance above filter background. Ela/rhabdomyosarcoma clone No. 1 and MDCK (control) epithelial cells had resistances of 250\(\Omega\)/cm\(^2\) and Ela/rhabdomyosarcoma clone No. 4.2 had a resistance of 204 \(\Omega\)/cm\(^2\). Ela induced the formation of tight junctions that could block ionic flow as efficiently as those in authentic epithelial cells.

Homotypic interactions between cadherins on adjacent epithelial cells can initiate junctional complex formation (e.g., Gumbiner et al., 1988; Geiger and Ayalon, 1992). Because of the large number of cadherin family members (Geiger and Ayalon, 1992) protein lysates of the Ela-expressing cell lines were initially probed with an antibody that recognizes all cadherins through a 21 aa conserved cytoplasmic epitope. Ela induction of a cadherin band was observed only in the RM cells (Fig. 7). This band was identified as E-cadherin by reaction with an E-cadherin-specific antibody (Fig. 7). A Northern blot probed with an E-cadherin probe confirmed

Figure 5. Analysis of the expression and localization of the tight junction protein ZO-1. (Left) Expression of ZO-1 protein in rhabdomyosarcoma cells and Ela derivatives: analysis by Western blotting. (Right) Immunofluorescent localization of the tight junction protein ZO-1 in control (vec/FS, ve/RM) and Ela-expressing (Ela/FS, Ela/RM) cells.
Figure 6. Dome formation and polarized distribution of Na-K ATPase in Ela-expressing RM cells. (a) Domes were identified by phase contrast microscopy as depicted in C and D; these represent different focal planes where cells around the top rim of the dome (C) or at the bottom surface (D) were in focus. A and B show confocal microscopy of a fluorescently labeled dome, viewing the xy plane (B) or along the xz plane (A). (b) The distribution of sodium-potassium ATPase in confluent cultures of RM or Ela/RM cells was visualized by staining with anthroyl-ouabain, followed by fluorescence microscopy, as described in Materials and Methods.

that E-cadherin mRNA was induced by Ela in the RM (Fig. 7) but not in the FS or RM2 cell lines (data not shown).

Induction of Other Epithelial Markers and Effects on Non-epithelial Markers

Because simple epithelial cells contain intermediate filaments composed of keratins type 8 and 18 (Oshima et al., 1983), Northern blots were probed to determine whether Ela induced these genes. Ela induced keratin 18 expression in RM and melanoma cells and induced keratin 8 expression in the RM cells (Fig. 8 a). Based on these results, keratin-containing intermediate filaments were expected to be found

Figure 7. Expression of cadherins in Ela-expressing cell lines. (Top) A Western blot containing protein lysates from parental (−) or Ela-expressing (+) cell lines was probed with pan-cadherin antibody or E-cadherin–specific antibody, as described in Materials and Methods; lysates from mammary carcinoma cell line mcf-7 and from the keratinocyte cell line HaCat were used as positive controls. (Bottom) The Northern blot, containing RNAs from parental (−) or Ela-expressing (+) RM cells was probed with an E-cadherin–cDNA probe.
Figure 8. Expression of keratins in Ela-expressing cell lines. (Left) The Northern blot, containing RNAs from parental (-) or Ela-expressing (+) RM or ML cells was probed with human keratin 18 (K18) or human keratin 8 (K8) probes, the expression levels were similar to those seen in HeLa cell control (data not shown). (Middle) Ela/RM clone 4.2, vec/RM (negative control) or mammary carcinoma mcf-7 cells were stained with anti-K18 antibody CK5 and photographed on a fluorescence microscope.
in the Ela-expressing rhabdomyosarcoma cells. Immunofluorescent antibody staining confirmed the presence of these intermediate filaments (Fig. 8b).

Ela did not induce gene expression globally. The gene for the glycolytic enzyme GAPDH was unaffected by Ela expression (Fig. 9), except in the osteosarcoma cells, where it was slightly induced.

To determine whether Ela repressed the expression of certain nonepithelial genes, RM2 cells were probed for desmin protein, cardiac α-actin mRNA and myoD mRNA (Fig. 9). The expression of these muscle-specific gene products was repressed by Ela. The melanocyte-specific tyrosinase gene was down-regulated in the melanoma cells as well. Ela also down-regulated the fibronectin gene, which is usually expressed at higher levels in mesenchymal than in epithelial cells.

Discussion

Programmed interconversions between epithelial cells and mesenchymal cells occur during development (Hay, 1992). An important goal is to identify the regulatory molecules and mechanisms involved in these interconversions. This may also contribute to an understanding of how the epithelial phenotype is compromised by oncogenic transformation. Because Ela is a viral gene, it is not normally involved in these interconversions. However, analysis of them may be facilitated by the use of Ela, as demonstrated by the results presented herein.

The adenovirus Ela gene was previously shown to be a tumor suppressor gene in various human cell lines (Frisch, 1991). Intuitively, a tumor suppressor gene might be expected to revert tumor cells to their pre-transformed phenotype. However, as reported herein, cell lines expressing Ela did not resemble their corresponding parental phenotypes; for example, melanoma cells that expressed Ela (Frisch, 1991) did not morphologically resemble melanocytes. Instead, the morphologies of Ela-expressing cell lines constructed in this laboratory, as well as those depicted photographically in the literature (Maruyama et al., 1987; Taylor et al., 1993) resembled that of epithelial cells, regardless of the parental cell type. The morphologic effects were clearly due to Ela expression rather than selection of spontaneously occurring variants for the following reasons: (a) all of the parental cell lines were subcloned prior to introduction of Ela; (b) after infection with the empty retrovirus vector and G418 selection, no epithelioid colonies were observed; (c) every clone that expressed Ela had an epithelioid morphology; (d) colonies of epithelioid morphology were observed within 3-5 d after infection, rendering the hypothetical requirement for secondary stochastic changes unlikely; and (e) microinjection of Ela expression plasmids into HT1080 induced desmosomes which were detectable by immunofluorescence two days after injection (data not shown).

The combined data in this report suggest that Ela is capable of programming several aspects of the epithelial phenotype. The effects of Ela appeared to reflect an epithelial-specific programming event rather than generalized activation of transcription. The 243-aa Ela protein used in this study is not a general activator of transcription (Shenk and Flint, 1991; Berk, 1986; Braithwaite et al., 1991). It did not affect the expression of a housekeeping gene (GAPDH), and it repressed the expression of non-epithelial genes, indicating the specificity of Ela's effects.

Certain genes have been shown previously to cause the loss of epithelial characteristics. Oncogenes such as c-fos (Reichmann et al., 1992) and v-src (Warren and Nelson, 1987) have been shown to cause the loss of epithelial characteristics. The effects of these oncogenes on epithelial cell morphology have been studied extensively, and it is known that they can induce a variety of morphologic changes. These changes include the loss of cell-cell adhesion, altered cell shape, and decreased cell migration.

Figure 9. Expression of housekeeping (GAPDH) or non-epithelial-specific genes (tyrosinase, desmin, cardiac α-actin, fibronectin [FN], myoD) in Ela-expressing cell lines. Western blots (desmin) or Northern blots (other panels) were reacted with the anti-desmin antibodies or the appropriate cDNA probes as described in Materials and Methods.
tion factor has been indentified to date, a different paradigm protein. In addition, because no epithelial-specific transcrip-
ture.

transcription factor have been widely reported in the liter-

cells. Genes encoding epithelial cell adhesion molecules re-

chymal cells in culture, especially the rhabdomyosarcoma

ferring an extensive epithelial commitment upon mesen-

lar fibroblast) a gene such as Ela could be of substantial

be involved in cell differentiation. The role of retinoblastoma

protein binding to Ela is unclear in this context. Although

emerges at around the time of compaction of the mamma-

sian eight-cell embryo, being the earliest identifiable embry-
onic cell type. It possesses intercellular adhesions involving

E-cadherin, tight junctions, and cytokeratin filaments. All

primary mesenchymal cells arise by differentiation from

these proto-epithelial cells; some of this mesenchyme will

convert into epithelia, which can in some cases be the

precursor of secondary mesenchyme (e.g., skeletal muscle)

(Hay, 1990; Fleming and Johnson, 1988; Birchmeier and

Birchmeier, 1993).

It may prove useful therefore to conceptualize the epithe-

lial cell as a "default phenotype" for mammalian cells. Under

this model, epithelial gene expression involves only the

interplay of ubiquitous transcription factors. Tissue-specific

transcription factors both activate appropriate tissue-specific
gene expression and repress epithelial-type gene expression

(e.g., muscle creatine kinase and keratin 18, respectively).

The model predicts that epithelial-type genes are under

repression in non-epithelial cells. A relevant example of this

is the epithelial-specific promoter of the human papilloma

virus p97 gene. Its epithelial transcription is driven purely

by "ubiquitous" activator proteins, including the factor NF-

1/CTF. Fibroblasts express both NF-1/CTF and an additional

NF-1 family member (called NF-IX) that represses transcrip-
tion through the NF-1 sites of the promoter (Apt et al.,

1993).

The default phenotype model suggests that the epithelial

conversion effects of Ela may largely reflect de-repression of

epithelial promoters resulting from inhibition of the expres-
sion or transcriptional activities of non-epithelial transcrip-
tion factors. Analysis of the interactions of Ela with cellular

proteins will reveal the first step of the process. The NH2

terminus of Ela interacts with several potentially global tran-

scriptional regulators. These include: (a) p300, a nuclear

DNA-binding phosphoprotein (reviewed in Moran, 1993);

(b) Dr-1, which itself interacts with TATA box binding pro-

tein (Inostroza et al. 1992); (c) helix-loop-helix domains of

(at least) myogenin and El2 (Taylor et al., 1993) which may

be involved in cell differentiation. The role of retinoblastoma

protein binding to Ela is unclear in this context. Although

this protein regulates the transcription factor E2F, the known

target genes for this factor are ubiquitously expressed, cell

cycle-related genes such as c-myc and DHFR (reviewed in

Nevins, 1992).

Although the significance of this work is primarily in the

molecular biology of cell differentiation, it also may be in-

formative to consider a new function of Ela for adenovirus.

Adenovirus has evolved to infect human airway epithelial

cells. However, it it infects a non-epithelial cell (e.g., tonsil-

lar fibroblast) a gene such as Ela could be of substantial

benefit to the virus. By altering the transcriptional ma-

chinery of the cell to (even modestly) activate epithelial-type

promoters, Ela would presumably stimulate the transcrip-
tion of adenovirus promoters even in the originally inap-

propriate host cell type. This would allow some probability

of viral life cycle completion.

In summary, the conversion into an epithelial phenotype

caused by Ela provides a new system for investigating the in-

tracellular mechanisms by which epithelial and mesen-

chymal cells interconvert during development (reviewed in

Hay, 1990). Because the phenotypic effects of Ela are medi-

ated by its interactions with a discrete set of cellular proteins
(Dyson and Harlow, 1992; Moran, 1993), it may be possible to use this phenomenon to molecularly analyze the epithelial–mesenchymal transition, albeit run in reverse. It also may provide a conceptual framework for understanding the diversity of transcriptional effects of Ela: by altering a master switch that converts a mesenchymal cell to an epithelial cell, the transcription of a large number of genes could be affected.

The tumor suppressive effect of Ela may result in part from its induction of intercellular adhesion molecules (Tsukita et al., 1993), which may provide a new approach for understanding and controlling cancer. In particular, epithelial cells containing junctional complexes are sensitive to anoikis, a form of apoptosis induced by the disruption of cell–matrix interactions (Frisch and Francis, 1994; Ruoslahti and Reed, 1994).

Finally, it is interesting that the cells of the mouse morula or blastocyst contain an Ela-like functional (i.e., transcriptional) activity (Suemori et al., 1988). This activity disappears upon implantation of the embryo. Conceivably, there is a cellular gene functionally analogous to Ela that maintains the epithelial phenotype during early development.

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