

"Trans-Differentiation" from Epidermal to Mesenchymal/Myogenic Phenotype Is Associated with a Drastic Change in Cell-Cell and Cell-Matrix Adhesion Molecules

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Abstract. Cells of the human keratinocyte line HaCaT were shifted to a mesenchymal/myogenic phenotype (DTHMZ cells) by MyoD1 transfection, 5-aza-2'-deoxycytidine treatment, and selection for reduced adhesion on plastic. Since this correlated with loss of stratification (inability to form a multilayered tissue), we determined the status of cell-cell and cell-matrix adhesion molecules involved in epidermal morphogenesis. Expression of desmosomal proteins (plakoglobin, desmoglein, desmoplakin) and uvomorulin was no longer detectable at the mRNA and protein level in the DTHMZ cells while both HaCaT cells and malignant variants (transfected with c-Ha-ras oncogene) expressed uvomorulin in vitro and in transplants in vivo, the latter even in invasively growing tumor nodules. Furthermore, HaCaT cells stained positive for the integrin subunits $\beta 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$, typical for cultured keratinocytes. In contrast, the putative fibronectin receptor $\alpha 5\beta 1$, common also in fibroblasts, was the only integrin showing strong staining in DTHMZ

cells. The integrin subunits αv and $\alpha 6$, clearly expressed at the mRNA level, weakly stained HaCaT cultures and led to a dotlike fluorescence in DTHMZ cells, possibly representing focal adhesion plaques. The respective integrin status correlated well with the growth behavior on different matrices. While HaCaT cells readily attached and proliferated on collagen (type I), fibronectin-coated, and laminin-coated collagen gels, DTHMZ cells formed monolayers only on fibronectin-coated collagen. This was, however, not sufficient to allow stratification in vivo. Altogether, the status of adhesion molecules in DTHMZ cells more likely reflects that seen in mesenchymal cells as compared to the pattern of keratinocytes displayed by HaCaT cells. Moreover, since the DTHMZ cells were clearly HaCaT descendants, the results support our hypothesis of a "trans-differentiation" process from an epidermal (HaCaT) to a mesenchymal/myogenic phenotype (DTHMZ).

FORMATION and maintenance of structure and function of a tissue depends on the regulated interaction of cells both with each other and with the extracellular matrix (ECM). In recent years, a number of cell-cell and cell-matrix adhesion molecules have been identified which are believed to be involved in its regulation. This hypothesis is based on changes observed in the distribution of the different molecules during embryogenic development (for reviews see Edelman, 1986; Takeichi, 1988). Here are of interest the different epithelial-mesenchymal transitions that occur during development. In neural crest development, for example, neural crest cells change from an epithelial to mesenchymal phenotype (Thiery et al., 1982) and during kidney development, mesenchymal cells shift to an epithelial phenotype giving rise to the mesonephric and metanephric tubules (Vestweber et al., 1985; Ekblom, 1989). In these "trans-

differentiation" processes characteristic changes in cell adhesion molecules (CAMs) are observed.

Among the best-characterized cell surface glycoproteins are N-CAM and the cadherins (E-, N-, P-, and L-cadherin). The latter are calcium-dependent adhesion molecules which mediate tight cell-cell connections. Recent transfection studies have shown that segregation of two cell types occurred when they either expressed two different CAMs (after transfection) or when they expressed the same CAM, but at different levels (Nose et al., 1988; Friedlander et al., 1989). This indicates that amounts and/or combinations of CAMs might be a strong factor in tissue formation and that therefore changes in CAM expression may have profound consequences for tissue regulation. In this paper we have focused on E-cadherin, also called uvomorulin (Kemler et al., 1989) or liver cell adhesion molecule (Gallin et al., 1983), since it is found exclusively in epithelial cells and lost during transition to the mesenchymal phenotype, e.g., during early development when mesoderm separates from ectoderm (for review see Takeichi, 1988).

1. *Abbreviations used in this paper:* 5-aza-CdR, 5-aza-2' deoxycytidine; CAM, cell adhesion molecule; ECM, extracellular matrix.

Another group of adhesion molecules are the integrins, a family of *trans*-membrane glycoproteins mediating cellular interaction with ECM proteins (Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti, 1988). The integrins are composed of an α and a β subunit (Elices et al., 1991, and references therein) and are grouped into subfamilies according to their β subunit (for review see Hynes, 1992). The $\beta 1$ and $\beta 3$ subfamilies bind ECM molecules. Some of the integrins, in particular those within the $\beta 1$ family, show specificity for distinct ECM components, which are also present in the basement membrane of the skin. The basement membrane, which represents the junction between epidermis and connective tissue (mesenchyme), provides the attachment site for the basal layer of the epidermis. Components of the basement membrane, known to be recognized by specific integrin receptors, are laminin recognized by $\alpha 6\beta 1$ (Sonnenberg et al., 1990) and possibly also by $\alpha 6\beta 4$ (Carter et al., 1990; Sonnenberg et al., 1991; Lee et al., 1992). Collagen type IV is preferentially bound by $\alpha 2\beta 1$ which also recognizes laminin (Elices and Hemler, 1989) and fibronectin is bound by $\alpha 5\beta 1$ (Pytela et al., 1985). Integrin $\alpha 3\beta 1$ is thought to bind to all three proteins (Wayner and Carter, 1987; Gehlsen et al., 1989), suggesting that single ECM components can be recognized by several cell adhesion receptors, thereby allowing cooperative interactions for cell attachment. By permitting interaction between cells and their ECM, the integrins are believed to play a crucial role in tissue formation and maturation as for example in the epidermis (Adams and Watt, 1990; Hertle et al., 1991).

The multilayered architecture of the epidermis facilitates studies of maturation-related changes in cell-cell and cell-matrix interactions. This stratified epithelium is formed by several distinct cell layers generated from the proliferative basal layer which is in direct contact with the basement membrane. As keratinocytes leave the basal layer and commence terminal differentiation, they disconnect from the basement membrane. In cell culture studies it was shown that leaving the basal layer was associated with a modulation of integrin function, followed by loss of expression of $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 5\beta 1$ (Adams and Watt, 1990), and based on this observation it was hypothesized that the same might account for the epidermis *in vivo*.

Starting from a differentiating human keratinocyte line (HaCaT cell line; Boukamp et al., 1988), we have recently isolated a cell population that displays a mesenchymal/myogenic phenotype via three experimental steps (Boukamp et al., 1992). First, HaCaT cells were transfected with the muscle determination gene MyoD1 (Davis et al., 1987). This resulted in the synthesis of muscle cell markers such as myosin, desmin, and vimentin without substantially altering epidermal differentiation properties (morphology, keratin profile) *in vitro* or epidermal morphogenesis *in vivo* (formation of an epidermis-like epithelium in surface transplants; Boukamp et al., 1988; Breitkreutz et al., 1991). Subsequent treatment of a MyoD1 transfectant with 5-aza-2'-deoxycytidine (5-aza-CdR) had little effect on the behavior of the cells *in vitro*, but caused aberrant tissue morphogenesis *in vivo*. The resulting multilayered epithelium was morphologically unstructured and undifferentiated. Interestingly, this was not accompanied by blocked expression of epidermal differentiation markers. Finally, a subpopulation of less adhesive cells was isolated from the 5-aza-CdR-treated MyoD1 transfectants (DTHMZ cells) which had lost all epidermal and most

epithelial characteristics in culture. Moreover, the shift to a mesenchymal/myogenic phenotype (e.g., fibroblastic morphology, transactivation of other endogenous myogenic determination genes) was further accompanied by their inability to stratify in surface transplants (Boukamp et al., 1992).

To determine whether this "*trans*-differentiation" process from epidermal to mesenchymal/myogenic phenotype with concomitant loss of stratification was correlated with changes in cell-cell and cell-matrix adhesion molecules, we examined several classes of cell surface proteins. We could demonstrate that desmosomal proteins forming the typical epidermal cell-cell adhesion contacts were no longer expressed in the DTHMZ cells. Similarly, uvomorulin, also absent in normal fibroblasts, was downregulated at the protein as well as the mRNA level in the DTHMZ cells. From the integrins tested here, the laminin and collagen type IV receptors ($\alpha 2\beta 1$ and $\alpha 3\beta 1$) were lost, leaving the fibronectin receptor ($\alpha 5\beta 1$) as the major cell-matrix adhesion receptor of the integrin family. Thus, while the status of the cell-cell and cell-matrix adhesion molecules in the HaCaT cells largely reflected the one generally observed in normal keratinocytes, the status of the DTHMZ cells more closely resembled that of mesenchymal cells. This further supports our hypothesis of a "*trans*-differentiation" process from an epidermal (HaCaT) to a mesenchymal/myogenic phenotype (DTHMZ) induced in the HaCaT cells by the above-mentioned experimental manipulations.

Materials and Methods

Cell Cultures

All cells were routinely grown in modified Eagle's MEM (Fusenig and Worst, 1975) supplemented with 10% FCS and antibiotics at 37°C, 5% CO₂, and 95% air in a humidified incubator, as described previously (Boukamp et al., 1988). The cells were passaged once a week at a split ratio of 1:10, and the medium was changed twice a week. For immunofluorescence, the cells were seeded on glass coverslips and fixed either when still subconfluent or at confluence.

Northern Blot Analysis

Cytoplasmic RNA was isolated from cells according to the NP-40 method of Maniatis et al. (1982). 20- μ g RNA samples were electrophoresed through 1.2% agarose, MOPS-2.2 M formaldehyde gels (Lehrach et al., 1977), and transferred onto GeneScreen Plus membranes (Du Pont Co., Wilmington, DE) which were then baked for 2 h at 80°C. The hybridization reaction was done using the protocol of Church and Gilbert (1984) with probes labeled with ³²P using the procedure described by Feinberg and Vogelstein (1984). Finally, the membranes were exposed to XAR films (Eastman Kodak Co., Rochester, NY) for up to 4 wk at -70°C. The probe for plakoglobin was kindly provided by Dr. W. W. Franke, the uvomorulin probe was generously supplied by Dr. W. Birchmeier, and the probes for the integrin subunits $\alpha 6$, $\alpha 5$, and $\beta 1$ were a gift from Drs. R. Tamura and V. Quaranta, and $\alpha 2$ from Dr. E. Klein.

Tumorigenicity Test

Tumor formation was assayed over an observation period of 6 mo after subcutaneous injection of 5×10^6 cells in 100 μ l culture medium into the interscapular region of 6-8-wk-old nude mice (Swiss/c nu/nu backcrosses).

Transplantation

A transplantation assay, originally established for mouse keratinocytes (Fusenig et al., 1983), was modified to the needs of human keratinocytes and adapted to athymic nude mice. Briefly, organotypic cultures were prepared as described previously (Boukamp et al., 1990a). Collagen gels (4 mg/ml) of rat tail collagen (type I) were mounted in Combi-ring dishes (CRD)

(Noser and Limat, 1987; purchased from Renner KG, Dannstadt, FRG) and 2×10^5 cells were seeded onto the collagen surface. After ~ 17 h, the medium covering the cells was drained, and the collagen chambers were covered with hatlike transplantation chambers (Renner KG). The whole unit was then transplanted onto the muscle fascia of the back of nude mice, as described (Boukamp et al., 1985; 1990b). Transplants were excised "en bloc," embedded in Tissue Tec (Lab. Tec. Prods, Naperville, IL), snap frozen in isopentane, precooled in liquid nitrogen, and stored in liquid nitrogen until cryostat sectioning.

Indirect Immunofluorescence

Cells grown on glass coverslips or cryostat sections of surface transplants (5–7 μm thickness) were washed in PBS (3×5 min) before being fixed in either ethanol (for uvomorulin), methanol (for integrins), or acetone (for desmosomal proteins) for 10 min at -20°C . They were then rinsed in PBS for 10 min and incubated with antibody solutions overnight in a moist chamber at room temperature. Antibodies used were mouse monoclonal antibodies against integrin subunits $\alpha 2$, $\alpha 3$, $\alpha 6$, and $\beta 1$ (kindly provided by Dr. E. Klein), $\alpha 5\beta 1$ (Biomol/Telios, Hamburg, FRG); a rat antiserum against $\alpha 6$ (a gift from Dr. A. Sonnenberg); mouse monoclonal antibodies against desmosomal proteins (desmoplakin, plakoglobin, desmoglein; all purchased from Progen, Heidelberg, FRG); and a rabbit antiserum against uvomorulin (kindly provided by Dr. A. Kemmler). After incubation with the primary antibody, the coverslips or tissue sections were washed in PBS (3×10 min) and incubated for an additional 45 min with an anti-mouse, anti-rabbit, and anti-rat gamma globulin coupled to FITC (Dianova, Hamburg, FRG), respectively. After another wash of 3×10 min in PBS followed by a rinse in 10 mM Tris-HCl buffer (pH 8.5), the coverslips were finally mounted onto slides with Aqua mount (Lerner Laboratories, New Haven, CT), and the tissue sections were covered with coverslips. Staining was then viewed under an inverted microscope equipped with epifluorescence optics (model IM 35; Carl Zeiss, Oberkochen, FRG).

Coating of Collagen Gels

Organotypic cultures (CRD chambers with collagen gels) were prepared as described above. 100 μl of fibronectin or laminin (100 $\mu\text{g}/\text{ml}$) was added onto the upper surface of collagen gels and incubated overnight. After three rinses in PBS 2×10^5 cells were seeded, incubated for 24 h, and the nonattached cells rinsed off and counted. For each substratum, two to three chambers were analyzed in two individual experiments.

Results

Loss of Desmosomal Proteins

The human keratinocyte line HaCaT, which gives rise to a

tightly packed and stratifying culture of typical cobblestone morphology (Fig. 1 A), was compared with a HaCaT derivative, the DTHMZ cells, that had acquired a fibroblastic morphology and exhibited less tightly packed monolayers even in long-term cultures (Fig. 1 B).

To determine whether this apparent change in cell contacts was associated with loss of epithelial cell–cell adhesion molecules, we first analyzed the presence of desmosomal proteins using antibodies specific for plakoglobin, desmoglein, and desmoplakin. While HaCaT cells synthesized all three proteins (desmoplakin: Fig. 2 A; desmoglein: Fig. 2 B; plakoglobin: Fig. 2 C), none of these components could be detected in DTHMZ cultures (Fig. 2, D–F). To determine whether this was due to insufficient translation or to downregulation at the transcriptional level, RNA was isolated and hybridized with a ^{32}P -labeled plakoglobin probe. This probe was used since plakoglobin shows the most wide-spread distribution among epithelial tissues and is occasionally also found in nonepithelial tissues (Koch et al., 1990). Therefore, it was the most likely component to be expressed by DTHMZ cells. While RNA from HaCaT cells gave a strong signal (Fig. 3, lane 1), no band was detected in RNA samples from DTHMZ cells (Fig. 3, lane 5). This was true for RNA samples derived from proliferating cultures (see Fig. 3, lanes 1–5) as well as from cultures maintained at confluence for an additional 5 wk (see Fig. 3, lanes 6–9) to allow maximal epidermal differentiation. Even after long-term exposure of the blots, no bands could be detected, suggesting a complete lack of transcription in these cells. In addition, we analyzed RNAs of HaCaT cell clones representing "intermediate steps" in the "trans-differentiation" process resulting in the DTHMZ cells, to determine whether downregulation of plakoglobin could be related to a specific treatment. HaCaT cells transfected with the vector plasmid (without MyoD-1 insert) expressed similar levels of plakoglobin as the parental cells (see Fig. 3, lanes 1, 2, and 6). Clone-13 cells, which represent an individually expanded clone of the MyoD1-transfected HaCaT cells and also clone-13aza cells, additionally treated with 5-aza-CdR, both still expressed plakoglobin although at a slightly reduced level as compared with HaCaT cells (see Fig. 3, lanes 3, 4, 7, and 8). This suggests that loss

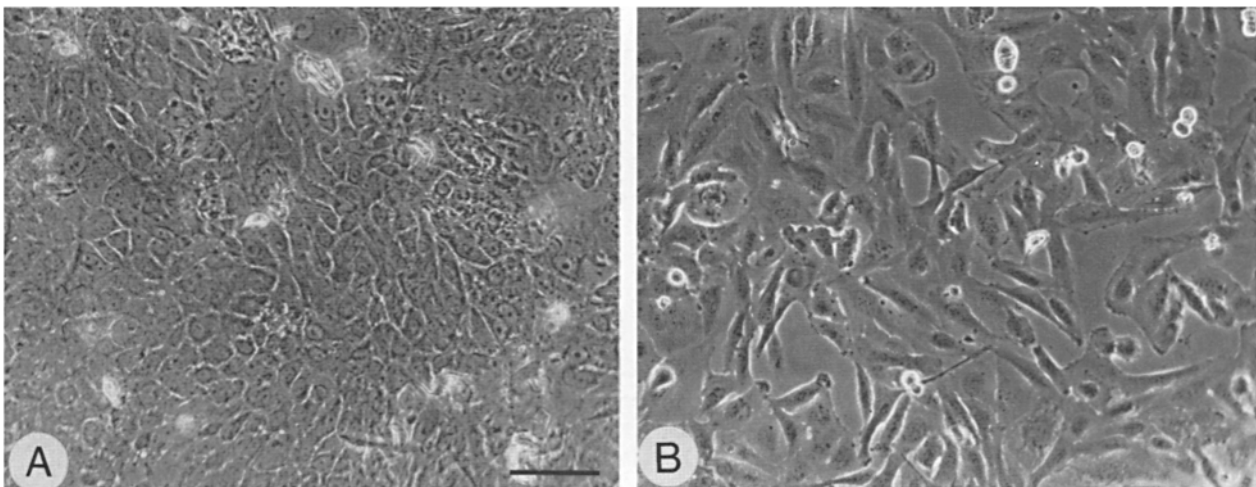


Figure 1. Phase-contrast micrographs of HaCaT and DTHMZ cells demonstrating the densely packed cobblestone pattern of the epidermal HaCaT cells (A) and the more loosely packed fibroblastic morphology of the DTHMZ cells (B). Bar, 100 μm .

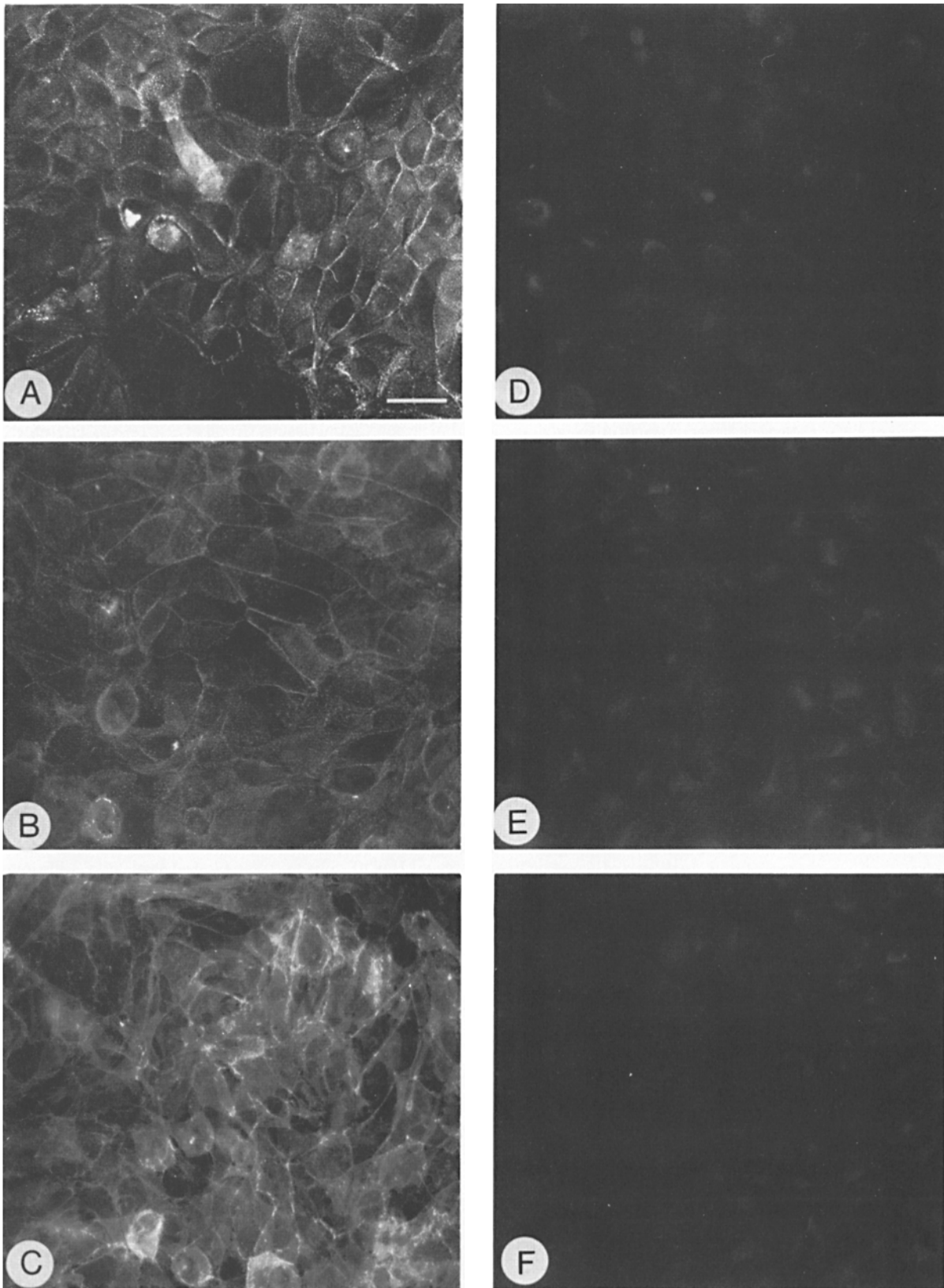


Figure 2. Immunofluorescence micrographs of HaCaT (*A–C*) and DTHMZ cultures (*D–F*) labeled with antibodies against desmoplakin (*A* and *D*), desmoglein (*B* and *E*), and plakoglobin (*C* and *F*). While the HaCaT cells show a characteristic dotlike staining around the cell membranes, the DTHMZ cells do not express any of these proteins. Bar, 25 μm .

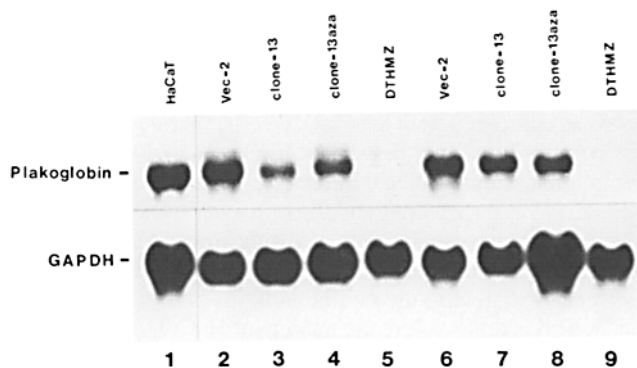


Figure 3. Northern blot analysis of mRNA from HaCaT cells and different HaCaT variants using a DNA probe for plakoglobin. Since DTHMZ cells were obtained after transfection with MyoD1 (clone-13) and additional treatment of the transfectant with 5-aza-CdR (clone-13aza), RNA from these clones in addition to transfection control cells (*Vec-2*) was examined to determine the step responsible for loss of plakoglobin expression in the DTHMZ cells. Proliferating (lanes 2–5) and postconfluent (5-wk-old) cultures (lanes 6–9) were analyzed. (Bottom) The same blot was hybridized with a 32 P-labeled GAPDH probe to evaluate the amount of loaded RNA.

of desmosomes was not a consequence of either MyoD1 transfection or 5-aza-CdR treatment, but required an additional step which resulted in reduced cell adhesion. Although it cannot be excluded that the reduced RNA level, seen in the HaCaT variants, was due to a preexisting negative subpopulation, it appears unlikely, since 100% of these cells stained positive for all three desmosomal components.

Loss of Uvomorulin

Another epithelium-specific cell–cell adhesion molecule is uvomorulin (Kemler et al., 1989). In examining expression of uvomorulin, we have used the mouse teratocarcinoma cell line F9 as a positive control (Fig. 4 A). Although the HaCaT cells were clearly positive, the characteristic membrane-bound staining was less intense (Fig. 4 B) as compared with that of the F9 cells which are known to express high amounts of the protein. The DTHMZ cells, on the other hand, were completely negative (Fig. 4 C). According to the desmosomal proteins, this was due to lack of transcription. Thus, while hybridization with a 32 P-labeled uvomorulin probe readily detected uvomorulin RNA in the HaCaT cells, none could be detected in the DTHMZ cells (Fig. 4 D). As seen with plakoglobin, the “precursor” cells (clone-13 and clone-13aza) were still positive for uvomorulin at the RNA and protein level (data not shown).

In several cell lines, loss of uvomorulin expression has been suggested to correlate with gain of invasive properties (Behrens et al., 1989; Chen and Öbrink, 1991; Frixen et al., 1991). We therefore analyzed HaCaT and DTHMZ cells for their invasive and tumorigenic capacity by surface transplantation and by injection, respectively. The HaCaT cells are nontumorigenic when injected subcutaneously into athymic nude mice and form a noninvasive well-stratified and differentiated epidermis-like epithelium in surface transplants (Boukamp et al., 1988; Breitkreutz et al., 1991). After transfection of HaCaT cells with the c-Ha-ras oncogene, we obtained tumorigenic clones (HaCaT-ras), some of which

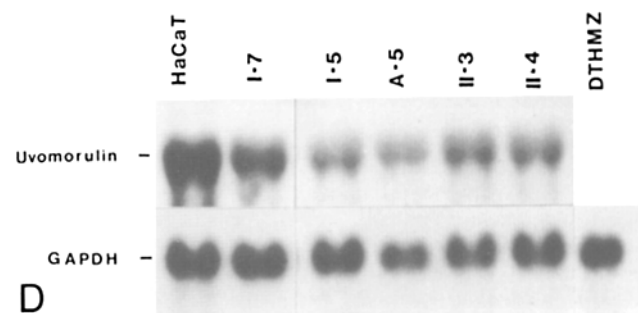
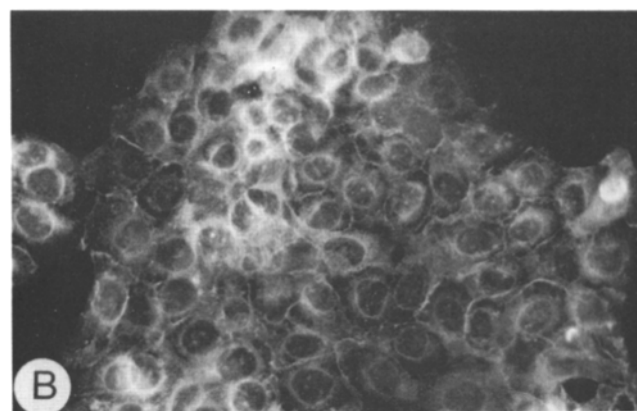
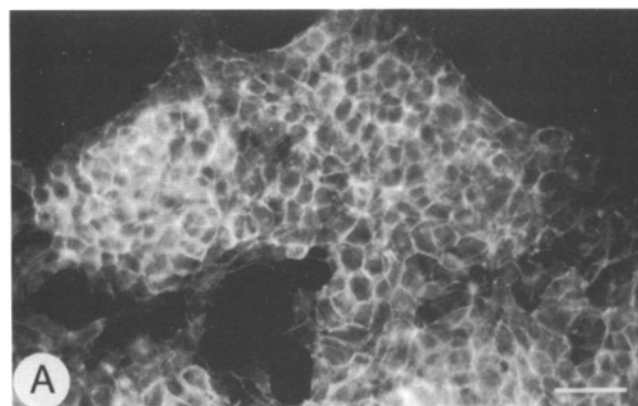


Figure 4. Uvomorulin expression in F9, HaCaT, and DTHMZ cells (A–C) analyzed by immunofluorescence microscopy. F9 cells (A) were used as positive control. While HaCaT cells clearly expressed uvomorulin (B) DTHMZ cells were completely negative (C). The same was true at the RNA level (D). Comparable with HaCaT cells, benign tumorigenic (c-Ha-ras oncogene transfected clones I-7, I-5, and A-5) and malignant variants (II-3 and II-4) expressed uvomorulin RNA. Bar, 25 μ m. (D) Same blot hybridized with a GAPDH probe, so as to control for loaded RNA.

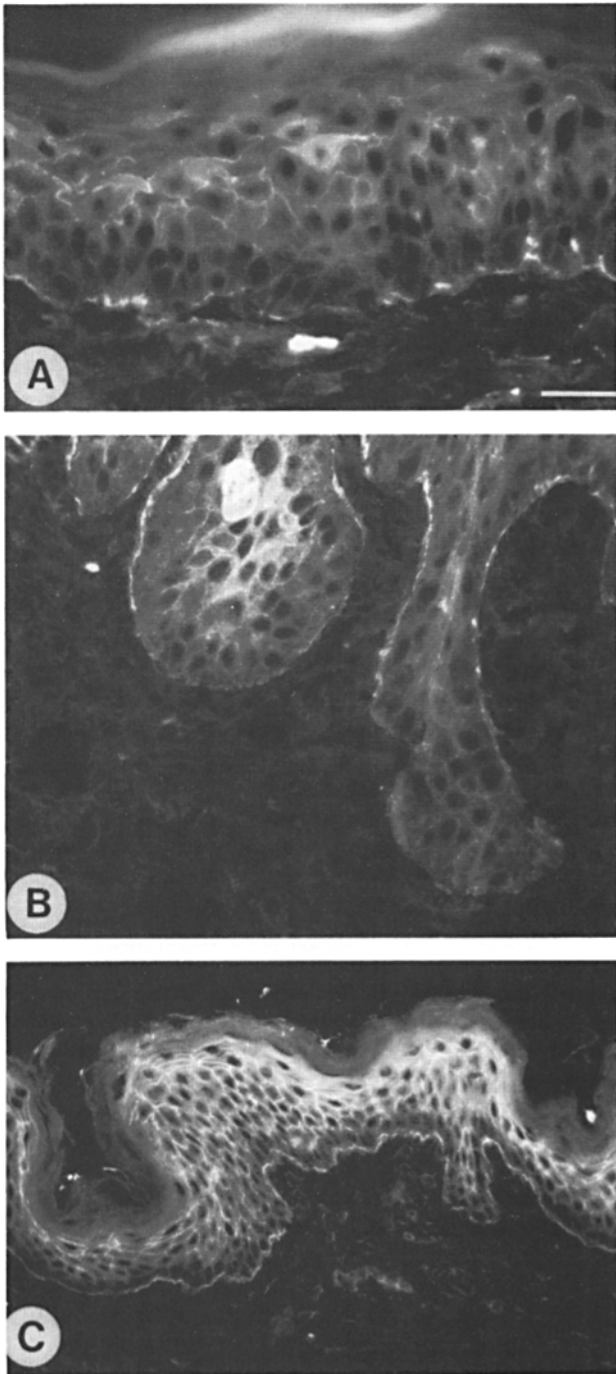


Figure 5. Distribution of uvomorulin (immunofluorescence microscopy) in surface transplants (*A* and *B*) and human skin (*C*). HaCaT cells and their malignant variants form well-organized and differentiated epidermis-like epithelia. In transplants of HaCaT cells (*A*) uvomorulin was predominantly expressed suprabasally. In the malignant HaCaT-ras clone II-3 (*B*), the protein was also present in areas invading the host tissue. In the epidermis (*C*), uvomorulin staining was most intense suprabasally, similar to that seen in transplants of the HaCaT cells. Bar, 25 μ m.

formed benign tumors after subcutaneous injection and remained noninvasive in surface transplants (clones I-7, I-5, and A-5), whereas other clones gave rise to malignant tumors (clones II-3 and II-4). In surface transplants the latter also started off as a well-differentiated surface epithelium but

rapidly invaded the underlying mesenchyme and formed tumorlike nodules (Boukamp et al., 1990b; Breitkreutz et al., 1991). In culture, all HaCaT-ras clones expressed uvomorulin RNA (Fig. 4 *D*). Although the RNA levels were reduced as compared with the HaCaT cells, there was no obvious correlation with a respective tumor phenotype (benign or malignant tumorigenic). Similarly, in transplants of both, the HaCaT cells (Fig. 5 *A*) and the invasive HaCaT-ras clones (Fig. 5 *B*), there was staining of uvomorulin. Although this was less intense than in normal skin (Fig. 5 *C*), the staining was clearly visible at the cell boundaries and, most importantly, also in areas where the malignant HaCaT-ras cells had invaded the host tissue (see Fig. 5 *B*).

On the other hand, DTHMZ cells, which were clearly negative for uvomorulin, were nontumorigenic (s.c. injection). In surface transplants they formed a monolayer of flat elongated cells and remained noninvasive (Boukamp et al., 1992). These data suggest that in the HaCaT system, loss of uvomorulin expression is not correlated with the acquisition of invasive capacity but rather associated with the "trans-differentiation" process from epidermal to mesenchymal/myogenic phenotype postulated for the DTHMZ cells.

Changes in Integrin Expression

The DTHMZ cell population was originally selected on the basis of reduced adhesion to plastic in conventional cultures (Boukamp et al., 1992). In many cases, it has been shown that integrin subunits $\alpha 5$ and αv are involved in the focal adhesions (for review see Burridge, 1986) representing the sites of initial attachment to the substratum. We therefore investigated the possibility that differences in the integrin status of HaCaT and DTHMZ cells were responsible for the observed changes in adhesion of the DTHMZ cells. Integrin subunits $\beta 1$, $\alpha 2$, and $\alpha 3$, typical for epidermis, were readily detectable in HaCaT cultures using integrin-specific antibodies (Fig. 6, *A*, *C*, and *E*). Integrin $\alpha 5\beta 1$, normally only seen in fetal skin as well as in primary cultures of keratinocytes (Sonnenberg et al., 1991), was also strongly expressed by HaCaT cells in vitro (Fig. 6 *G*). In contrast, αv , the subunit of the classical vitronectin receptor ($\alpha v\beta 3$) which is common in focal adhesions (together with $\alpha 5\beta 1$), was only faintly expressed by the HaCaT cells (Fig. 6 *J*). Another integrin subunit expressed by keratinocytes is $\alpha 6$ (complexed with $\beta 4$). This integrin is apparently an integral part of hemidesmosomes (Stepp et al., 1990; Sonnenberg et al., 1991), adhesion structures connecting epidermal basal cells with the basement membrane. When HaCaT cells were labeled with an antibody against $\alpha 6$, staining appeared to be intracellular and was most intense in the perinuclear region (Fig. 6 *L*).

While DTHMZ cells still expressed $\beta 1$ (Fig. 6 *B*) and $\alpha 5$ integrin subunits (Fig. 6 *H*), similar to cultured fibroblasts (Fig. 6 *I*), no staining could be detected for $\alpha 2$ and $\alpha 3$ subunits (Fig. 6 *D* and *F*). Antibodies against αv gave rise to a weak but distinct dotlike fluorescence (Fig. 6 *K*), as did antibodies against $\alpha 6$ (Fig. 6 *M*). Thus, besides weak expression of αv and $\alpha 6$, the DTHMZ cells revealed only strong expression of the integrin subunits $\beta 1$ and $\alpha 5$. This indicates that the putative fibronectin receptor ($\alpha 5\beta 1$) represents a major integrin in these cells.

At the mRNA level $\alpha 5$ (Fig. 7) and $\beta 1$ (data not shown) were similarly expressed by HaCaT and DTHMZ cells and

all DTHMZ "precursor" cells (Vec-2, clone-13, clone-13-aza). Similarly, blots hybridized with probes for αv and $\alpha 6$ showed comparable levels of RNA for all variants (Fig. 7). An unexpected result was obtained with a probe for the integrin subunit $\alpha 2$. While $\alpha 2\beta 1$ could not be detected at the protein level, the DTHMZ cells clearly expressed $\alpha 2$ mRNA (Fig. 7) suggesting that lack of protein was due to downregulation at the posttranslational level.

Matrix-dependent Growth Behavior

To study growth *in vivo*, the cells are plated onto collagen gels (type I) which provide the bottom of the transplantation chambers. The apparent loss of expression of integrin subunits $\alpha 2$ and $\alpha 3$ in DTHMZ cells might indicate that collagen is not the adequate matrix. Furthermore, to exclude that loss of stratification in surface transplants was due to inappropriate experimental conditions we determined growth behavior of HaCaT and DTHMZ cells on different substrata *in vivo*. According to transplantation, the cells were grown in transplantation chambers (with the cells exposed to the air). In addition, in some chambers the collagen was coated with laminin or fibronectin. When attachment was measured 24 h after plating (number of unattached cells counted), the HaCaT cells had attached well on all matrices with an attachment rate of $\sim 95\%$ for uncoated collagen and laminin-coated collagen, and $\sim 98\%$ for fibronectin-coated collagen. For DTHMZ cells the attachment rates were reduced with $\sim 85\%$ for laminin- and fibronectin-coated collagen, and $\sim 80\%$ for uncoated collagen.

In regard to spreading, HaCaT cells formed a tightly packed monolayer and started to stratify on all three matrices within 24 h (Fig. 8 A). For the DTHMZ cells, fibronectin provided the most favorable matrix. Whereas cells plated on fibronectin were well spread after 24 h and had formed a near-confluent cell layer (Fig. 8 D), cells plated on uncoated collagen (Fig. 8 B) or laminin (Fig. 8 C) were still mostly rounded up and cell-free areas were frequent (see arrowheads in Fig. 8, B and C). This discrepancy became even more pronounced 3 d after plating. HaCaT cells had already formed several cell layers (Fig. 8 E) while DTHMZ cells were able to establish a confluent monolayer only on fibronectin-coated collagen gels (Fig. 8 H). On collagen (Fig. 8 F) and laminin (Fig. 8 G), cells were still poorly spread and showed no signs of subsequent growth; i.e., cell-free areas were still frequent (see arrowheads in Fig. 8, F and G). Thus, although attachment was not significantly influenced by the different matrices, they clearly had an effect on the growth behavior of the DTHMZ cells suggesting that integrin $\alpha 5\beta 1$ was responsible for spreading and growth while it did not seem to be strongly involved in initial attachment.

Since coating of collagen gels with fibronectin considerably improved attachment and growth of DTHMZ cells *in vitro*, we also used fibronectin-coated collagen gels in surface transplants. However, the altered substratum did not influence tissue morphogenesis, since, also under these conditions, only monolayers of flat elongated cells were detected in transplants for up to 4 wk (data not shown). Thus, improved spreading and growth, as seen on fibronectin-coated collagen gels *in vitro*, was not sufficient for induction of stratification *in vivo*.

Discussion

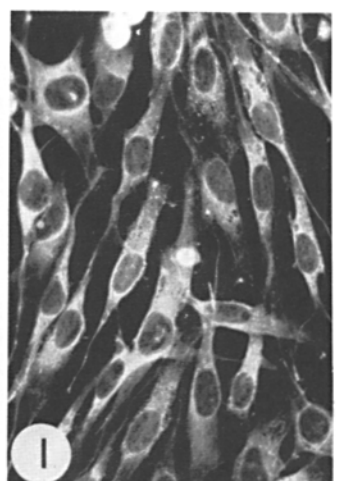
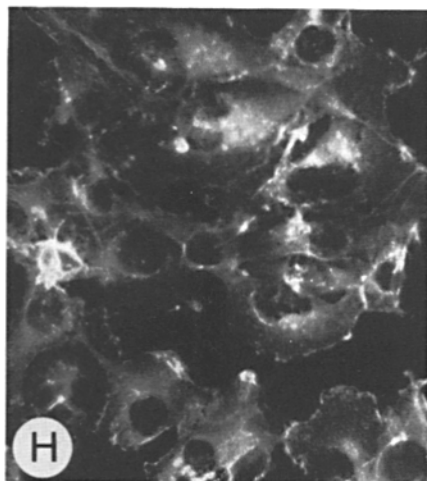
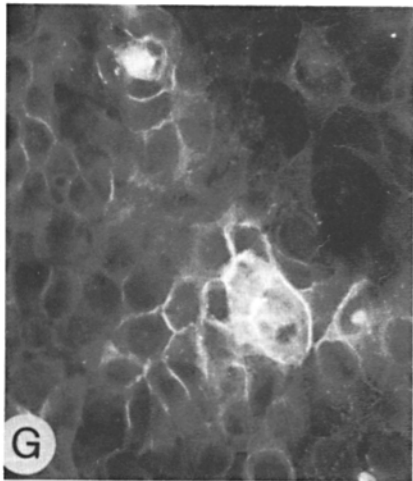
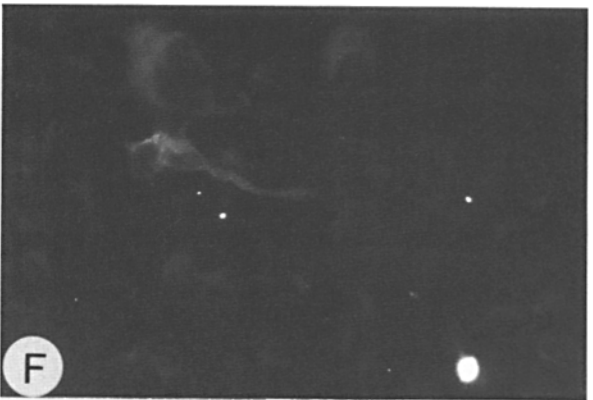
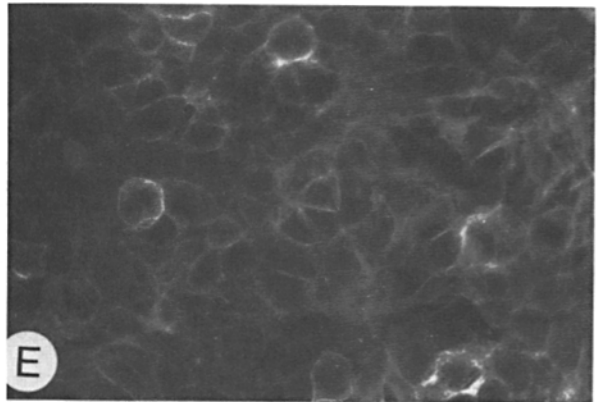
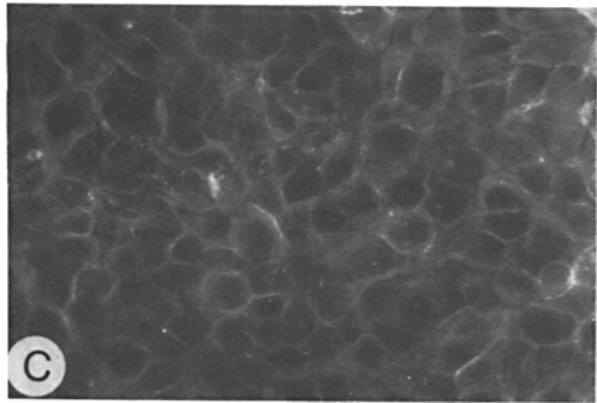
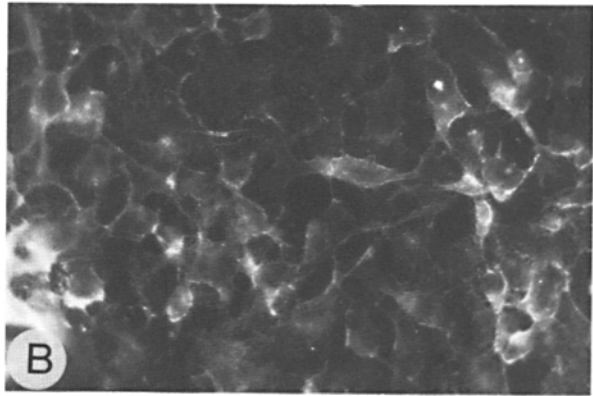
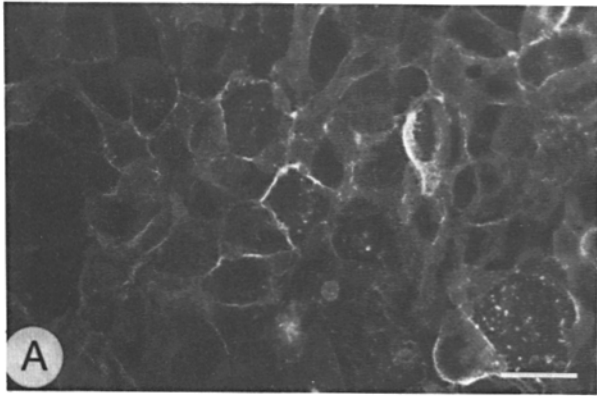
We have shown previously that transfection of the human keratinocyte line HaCaT with the muscle determination gene MyoD1 and additional treatment with 5-aza-CdR led to induction of muscle markers in the epidermal cells without severely abrogating epidermal differentiation (Boukamp et al., 1992). A subfraction of these cells, selected on the basis of reduced adhesion, revealed drastic changes in phenotype such as altered culture morphology (from a cobblestone to a fibroblastic appearance) and most strikingly loss of stratification, a very stable trait of the epidermal HaCaT cells (Boukamp et al., 1988; Ryle et al., 1989; Breitkreutz et al., 1991). Since these changes could have been the result of alterations in cell adhesion molecules, we have analyzed the status of different molecule classes in the parental epidermal HaCaT and the mesenchyme-like DTHMZ cells.

One type of calcium-activated cell adhesion complex which is responsible for the tight adhesion of keratinocytes in the three-dimensional tissue architecture of stratified epithelia are the desmosomes (for review see Schwarz et al., 1990). They are plaque-bearing junctions which are intercellularly anchored to keratin filaments, the epithelial-type intermediate filaments. While two of three desmosomal components tested here, desmoplakin and desmoglein, are highly specific for desmosomes, the third, plakoglobin, is also common in other adhesion plaques (Koch et al., 1990, and references therein). All three components were expressed by the parental HaCaT cells, whereas the DTHMZ cells did not show staining for any of these proteins. Furthermore, using probes for plakoglobin as the most widely distributed plaque protein, we determined whether loss of expression in DTHMZ cells occurred at the mRNA level. Since the HaCaT cells showed most intense staining for plakoglobin in dense and multilayered cultures, we analyzed RNA from exponentially growing as well as postconfluent cultures where differentiation was most pronounced. As expected, HaCaT cells expressed plakoglobin RNA under both conditions, whereas no signal was detectable at any time point for the DTHMZ cells, indicating completely down-regulated transcription.

Interestingly, during embryogenesis when the epidermis is still a single-layered epithelium (as early as 10 wk of gestation) the cells already express desmoplakin and plakoglobin (Boukamp, P., and I. Moll, manuscript in preparation). Thus, loss of expression of these proteins is not a mere consequence of loss of stratification but is possibly related to loss of epidermal differentiation.

Another fundamental adhesion molecule in epithelial cells, involved in cell-cell adherence, is uvomorulin (E-cadherin: Gallin et al., 1983; Kemler et al., 1989). This cell surface glycoprotein is present early in development already at the compaction of blastomeres and is functionally related to initial boundary formation, embryonic induction and migration, tissue stabilization, and regeneration (for review see Edelman, 1986). In the epidermis, uvomorulin is most strongly expressed in the suprabasal cell layers. In this context it is of interest to note that desmoglein, the constitutive component of desmosomes, is also a member of the cadherin family (Koch et al., 1990).

In contrast to epithelial cells, mesenchymal cells do not express such cell-cell adhesion molecules, and the transition



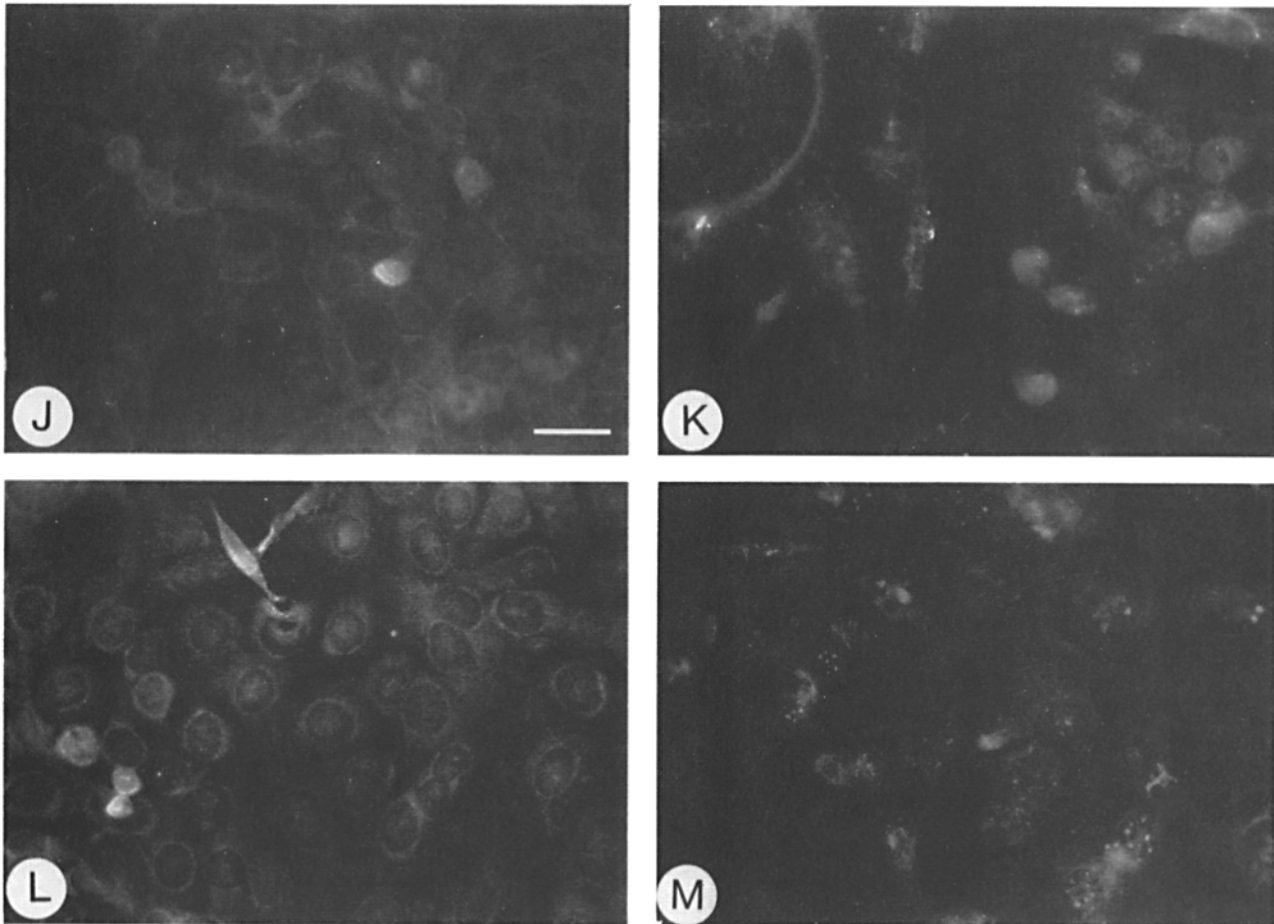


Figure 6. Immunofluorescence study of integrin subunit expression in cultures of HaCaT (*A, C, E, G, J, and L*), DTHMZ cells (*B, D, F, H, K, and M*), and normal human fibroblasts (*I*). Antibodies against $\beta 1$ labeled both HaCaT (*A*) and DTHMZ cells (*B*). While $\alpha 2$ and $\alpha 3$ were expressed by the HaCaT cells (*C* and *E*) but not by DTHMZ cells (*D* and *E*), $\alpha 5\beta 1$, the putative fibronectin receptor, was expressed by both HaCaT (*G*) and DTHMZ cells (*H*) as well as by normal fibroblasts (*I*). αv (*J*) was only faintly expressed by HaCaT cells (*J*) while staining for $\alpha 6$ appeared to be intracellular, being more intense in the perinuclear region (*L*). In the DTHMZ cells, staining for both integrin subunits resulted in a dotlike pattern (*K* and *M*). Bars, 25 μm .

from epithelial to mesenchymal phenotype during embryonic development is generally correlated with suppression of these genes and with very few exceptions, such as urogenital structures, this status is constantly maintained later on (for review see Edelman, 1986). Comparable with normal keratinocytes, uvomorulin was expressed by the epidermal HaCaT cells. However, it could not be detected in the DTHMZ cells, neither at the protein nor at the RNA level. Thus, loss of uvomorulin expression together with loss of desmosomal proteins in the DTHMZ cells provides additional evidence for our hypothesis that we had experimentally induced a “*trans*-differentiation” process in the epidermal HaCaT cells and that this was causally related to the failure of the DTHMZ cells to stratify, i.e., to form a multilayered tissue.

Recently, a number of reports discussed the correlation of loss of uvomorulin expression and acquisition of invasive properties in epithelial tumor cells (Behrens et al., 1989; Chen and Öbrink, 1991; Frixen et al., 1991; Sommers et al., 1991). In this context it was postulated that uvomorulin acts as an “invasion suppressor” molecule (Vleminckx et al., 1991). Therefore we investigated whether loss of uvomorulin expression in the DTHMZ cells had resulted in a tumori-

genic (after s.c. injection into nude mice) and invasive (in surface transplants) phenotype. However, like in the parental HaCaT cells both traits were absent in the DTHMZ cells. On the other hand, we recently described HaCaT cell populations which were obtained after c-Ha-ras oncogene transfection and which had become tumorigenic and invasive (Boukamp et al., 1990b). These cells were perfectly able to form a well-differentiated surface epithelium upon transplantation (Boukamp et al., 1990b; Breitkreutz et al., 1991) but additionally invaded the collagen matrix and eventually the mouse mesenchyme. The cells expressed uvomorulin RNA in culture and the protein was also detected in vivo in transplants even in invasively growing tumorlike nodules. This finding differs from a recent report by Mareel et al. (1991) showing that uvomorulin was downregulated in vivo in tumors as a response to the host environment. Thus, in our model system of differentiating human keratinocytes, loss of uvomorulin expression did not correlate with acquisition of the malignant phenotype but instead correlated with the transition from an epidermal to a mesenchymal phenotype. This is compatible with the view that uvomorulin acts as an inducer of cell surface polarity and of a corresponding selective distribution of cytoplasmic and membrane proteins in

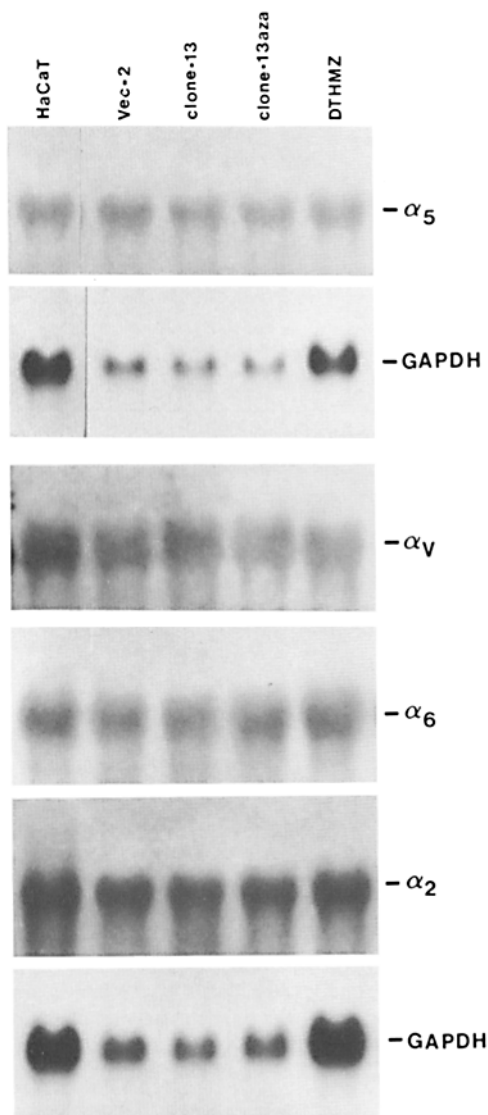


Figure 7. mRNA integrin status: Northern blot analyses of total RNA isolated from HaCaT, Vec-2, clone-13, clone-13aza, and DTHMZ cells. The blots were hybridized with ^{32}P -labeled probes for the integrin subunits α_5 , α_v , α_6 , and α_2 as well as GAPDH as a control for the concentrations of loaded RNA. All integrin subunits were similarly expressed by all HaCaT variants.

epithelial cells, as recently suggested by McNeill et al. (1990).

A similar correlation was described for another surface marker supposedly involved in cell-cell and cell-matrix interaction (Inki et al., 1992, and references therein). Expression of this proteoglycan, called syndecan, was associated with epithelial differentiation during neoplastic growth; i.e., it was lost in poorly differentiated tumors or anaplastic spindle cell carcinomas while expressed in well-differentiated tumors (Inki et al., 1992). Furthermore, in a mouse mammary tumor cell model where steroid treatment induced a change from epithelial to mesenchymal morphology, this switch in phenotype was associated with reduced binding to the substrate and loss of syndecan expression (Leppä et al., 1991, 1992). Thus, there are obviously several surface molecules which maintain the complex three-dimensional struc-

ture of the stratified epithelial tissues and they are absent in mesenchymal cells and epithelial cells which have lost differentiation. Whether loss of these molecules are the initial events causing loss of differentiation or are consecutive effects, remains to be seen.

Integrins, the predominant family of cell surface receptors for ECM and basement membrane components, represent another set of cell surface glycoproteins believed to be involved in tissue organization and regulation. Several members of the family are found to be expressed in the epidermis (Konter et al., 1989; Hertle et al., 1991) and in cultured keratinocytes (Adams and Watt, 1990). Similarly, the epidermal HaCaT cells expressed integrin subunits β_1 , α_2 , α_3 , and α_5 as could be shown at the mRNA and protein level. Furthermore, this correlates well with recent radioimmunoprecipitation studies which showed that $\alpha_2\beta_1$ and $\alpha_3\beta_1$ but not $\alpha_1\beta_1$ were synthesized by the HaCaT cells (Scharffetter-Kochanek et al., 1992). They also expressed α_v and α_6 mRNA, and by immunofluorescence the proteins appeared to be intracellularly localized. Whether this pattern is due to incomplete posttranslational processing of the protein remains to be seen. In general, α_v is supposed to form a heterodimer with β_5 which is, however, not detectable in the skin. Whether it can associate with β_1 in the epidermis, as recently described for a human embryonic kidney cell line (Bodary and McLean, 1990), and in this configuration acts as a receptor for fibronectin, as proposed by Vogel et al. (1990), is still not clear.

The DTHMZ cells, on the other hand, were negative when labeled with antibodies against α_2 and α_3 integrin subunits. Surprisingly, transcription of α_2 was not downregulated as shown in Northern blot analyses. This suggests that expression of the α_2 subunit was posttranslationally regulated in the DTHMZ cells. Whether this indicates that the protein can be expressed under certain experimental conditions remains to be elucidated. In cultured fibroblasts integrin $\alpha_2\beta_1$ is expressed and seems to serve as a collagen-specific receptor while in other cell types $\alpha_2\beta_1$ can additionally function as a laminin receptor (Élices and Hemler, 1989).

While $\alpha_5\beta_1$ was further visible by immunofluorescence as a membrane-associated and intracellular staining, a dotlike distribution was seen for α_v and α_6 in the DTHMZ cells. Since expression of α_v and α_6 was additionally substantiated by Northern blot analyses the dots might well represent focal adhesion sites present on the lower surface of the cells. Thus, $\alpha_5\beta_1$ was the only one of the tested integrins which remained strongly expressed by the DTHMZ cells at the protein level and was also shown to be the only one of these integrins positive in fibroblasts in skin (Konter et al., 1989). Studies on the integrin protein pattern of muscle cells showed that β_1 and other β subunits are expressed (Clyman et al., 1990; Virtanen et al., 1990; Goodman et al., 1991) while the identity of α subunits is still a matter of debate. In developing skeletal and cardiac muscle cells none of the known α subunits could be detected (Virtanen et al., 1990). In murine myoblasts, on the other hand, $\alpha_v\beta_3$ was present (Goodman et al., 1991) and in muscle tissue both isoforms (A and B) of α_6 could be identified (Tamura et al., 1991). Thus, unless the integrin status of myogenic cells is defined in more detail, it remains unclear whether the DTHMZ cells more closely resemble fibroblasts or myogenic cells.

The drastic changes in adhesion molecules were assumed

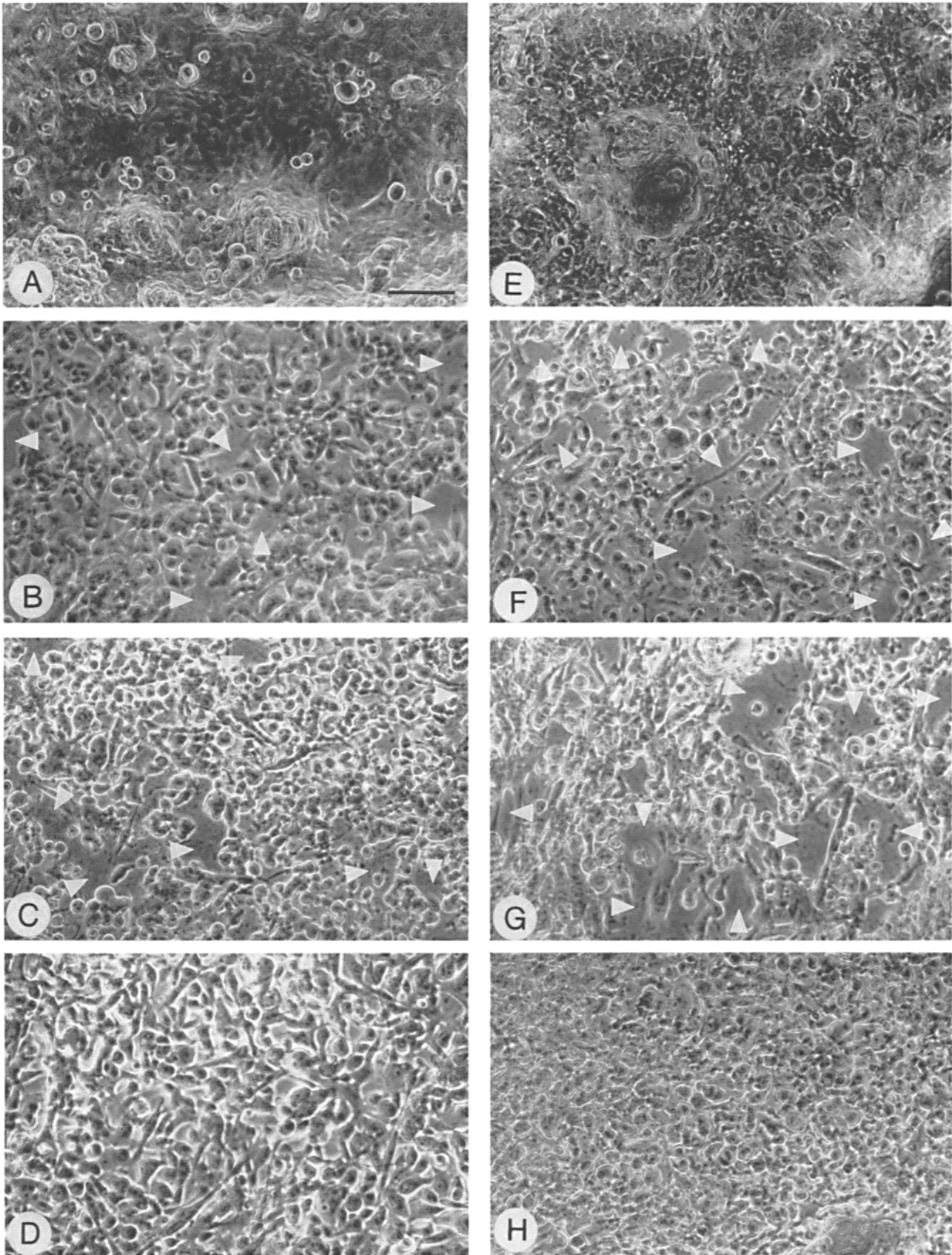


Figure 8. In vitro growth behavior on different matrices. HaCaT and DTHMZ cells were plated on gels of collagen type I (A, B, E, and F) or on collagen gels coated with either laminin (C and G) or fibronectin (D and H) and analyzed at 24 h (A–D) and 3 d (E–H) after plating. After 24 h, HaCaT cells had already formed a dense cell layer (A). The DTHMZ cells attached well on collagen type I (B) and laminin-coated collagen (C), but remained largely rounded up, leaving cell-free areas (arrowheads). Spreading was best on fibronectin-coated collagen (D) where the cells had covered the whole chamber within 24 h. 3 d after plating, the HaCaT cells had formed a multilayered tissue-like structure (E). The DTHMZ cells were still rounded up and had not further proliferated on collagen (F) and laminin (G) as indicated by the presence of still cell-free areas (arrowheads) while the cells had completely covered the bottom of the chamber and had formed a dense cell layer on fibronectin (H). Bars, 100 μm .

to have profound effects on cell attachment, spreading, and growth of the DTHMZ cells on different matrices. Epidermal HaCaT cells attached equally well to uncoated collagen (type I), or laminin- or fibronectin-coated collagen; and they formed multilayered tissue-like structures. For DTHMZ cells, on the other hand, attachment was reduced on all three matrices with the tendency that laminin-coated collagen might provide a slightly better substratum for initial attachment (measured 3 h after plating) while after 24 h the attachment rate was rather similar for all three substrates. How this correlates with the integrin status of the DTHMZ cells is unclear at present.

Preliminary blocking experiments with RGD peptides gave similar results. The RGD recognition site is located in the central binding domain of $\alpha 5\beta 1$ (Pierschbacher and Ruoslahti, 1984), which was shown to promote cell adhesion to fibronectin (Obara et al., 1988). Accordingly, addition of RGD peptides to keratinocytes in suspension resulted in a drastic decrease in attachment to fibronectin-coated plastic (Adams and Watt, 1990). However, under our conditions, addition of RGD peptides did not decrease attachment of the HaCaT cells, irrespective of the substratum used. This could either be explained by $\alpha 2\beta 1$ - and/or $\alpha 3\beta 1$ -mediated (RGD-independent) adhesion to still-exposed collagen fibers or by binding to other receptors not tested here. In the case of DTHMZ cells, addition of RGD peptides did reduce attachment to uncoated collagen and fibronectin-coated collagen while on laminin-coated collagen attachment remained largely unchanged. These findings might suggest that $\alpha 5\beta 1$ is only partly involved in the initial attachment of the DTHMZ cells. Moreover, because of the lack of principal laminin receptors ($\alpha 2\beta 1$ and $\alpha 3\beta 1$), attachment might be mediated by adhesion molecules other than those analyzed here.

In contrast to attachment, spreading of the DTHMZ cells correlated well with the maintained expression of $\alpha 5\beta 1$. While the cells largely remained rounded up on collagen (type I) or laminin-coated collagen and did not proliferate as indicated by several cell-free areas even 3 d after plating, they readily spread out on fibronectin-coated collagen gels and proliferated to form a dense cell layer. Thus, in our system the putative fibronectin receptor $\alpha 5\beta 1$ did seem to be important for spreading and proliferation. This suggests involvement of $\alpha 5\beta 1$ (either directly or indirectly) in the signal transduction pathway of the DTHMZ cells to mediate growth under the described conditions.

In conclusion, the data provide further evidence for the induction of a complex "trans-differentiation" process in the epidermal HaCaT cells which resulted in expression of a mesenchymal/myogenic phenotype. This "trans-differentiation" was associated with a drastic loss of cell-cell adhesion molecules (desmosomes, uvomorulin) as well as distinct losses in the principal cell-matrix adhesion molecules (integrins). The predominant expression of $\alpha 5\beta 1$ in the DTHMZ cells, also compatible with a mesenchymal phenotype, correlated with the ability of the cells to spread and proliferate on fibronectin-coated collagen but was insufficient to allow stratification to form a multilayered tissue.

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