Progressive Stages of "Transdifferentiation" from Epidermal to Mesenchymal Phenotype Induced by MyoD1 Transfection, 5-aza-2'-deoxycytidine Treatment, and Selection for Reduced Cell Attachment in the Human Keratinocyte Line HaCaT

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Abstract. The ability of the myogenic determination gene (MyoD1) to convert differentiating human keratinocytes (HaCaT cell-line) to the myogenic pathway and the effect of MyoD1 on the epidermal phenotype was studied in culture and in surface transplants on nude mice. MyoD1 transfection induced the synthesis of myosin, desmin, and vimentin without substantially altering the epidermal differentiation properties (morphology, keratin profile) in vitro nor epidermal morphogenesis (formation of a complex stratified squamous epithelium) in surface transplants, demonstrating the stability of the keratinocyte phenotype. 5-Aza-CdR treatment of these MyoD1-transfected cells had little effect on the cultured cells but a morphologically unstructured epithelium was formed with no indications of typical cell layers including cornification. Since prevention of epidermal strata in transplants was not accompanied by blocked epidermal differentiation markers (keratins K1 and K10, involucrin, and filaggrin), the dissociation of morphogenesis and expression of these markers argues for independently controlled processes. A subpopulation of less adhesive cells, isolated from the 5-aza-CdR treated MyoD1-transfectants, had lost most epithelial characteristics in culture (epidermal keratins, desmosomal proteins, and surface-glycoprotein Gp90) and had shifted to a mesenchymal/myogenic phenotype (fibroblastic morphology, transactivation of Myf3 and myogenin, expression of myosin, desmin, vimentin, and Gp130). Moreover, the cells had lost the ability to stratify and remained as a monolayer of flat elongated cells in transplants. These subsequent changes from a fully differentiated keratinocyte to a mesenchymal/myogenic phenotype strongly argue for a complex "transdifferentiation" process which occurred in the original monoclonal human epidermal HaCaT cells.

During embryogenesis the epidermis develops from a two-layered to a complex-stratified squamous epithelium. As the cells migrate from the basal proliferative zone they pass through an ordered sequence of defined morphological changes (histodifferentiation), leading to the formation of distinct strata. Concomitantly, specific differentiation products are sequentially expressed and these serve as excellent markers to determine the respective state of differentiation in experimental models. As part of this differentiation program, the synthesis of a number of proteins is induced including keratins, involucrin, and filaggrin. The keratins are the most abundant proteins in the keratinocytes and the position of a cell in the differentiation sequence is reflected by the composition of these intermediate filaments (Fuchs and Green, 1980; Moll et al., 1982*a*; Skerrow and Skerrow, 1983; Sun et al., 1983; Bowden et al., 1987). Poly- and monoclonal antibodies are available that are highly specific for the respective keratins and thus label discrete compartments, allowing for the evaluation of the status of differentiation. Involucrin, a precursor of the cornified envelopes in the outermost epidermal layers, marks a later stage in epidermal differentiation (Rice and Green, 1979; Watt and Green, 1981). Similarly, filaggrin, the major matrix component of the keratohyalin granules appears in the stratum granulosum, the zone before the keratinocytes lose cell organelles and become dead horn-squames (Dale et al., 1978; Scott and Harding, 1981).

Many details are known for the identification of the epidermal differentiation process and the different components are well characterized. Little, however, is known about the genes responsible for the ordered sequence of expression of the different markers and thus the regulation of differentiation. In myogenesis, on the other hand, four such genes have been described. These are MyoD1 (Davis et al., 1987), myogenin

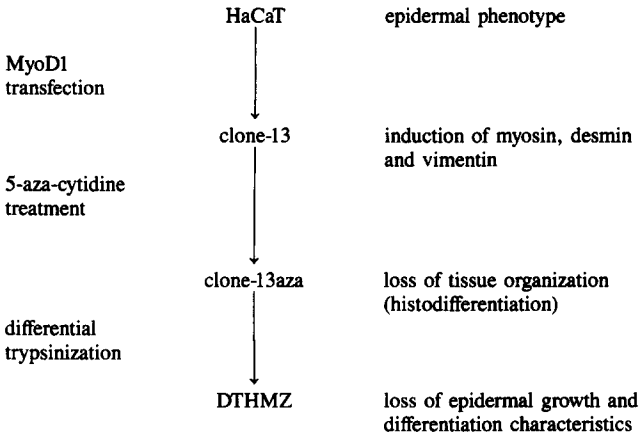
(Wright et al., 1989), Myf5 (Braun et al., 1989), and Mrf4 (Rhodes and Konieczny, 1989), also known as herculin (Miner and Wold, 1990), and Myf6 (Braun et al., 1990). These genes, when transfected into a number of nonmyogenic cell types, can convert them into stable myogenic clones which then express reporter genes normally inactive in these cells (for review see Olson, 1990). Certain cells of epithelial origin, such as HeLa or HepG2, appear to be refractory to myogenic conversion (Weintraub et al., 1989; Schäfer et al., 1990) and it has been hypothesized that the failure of MyoD1 to activate myogenesis in these cases is due to negative factors present in some cell types or that factors with which MyoD1 cooperates are not ubiquitous (Olson, 1990). Alternatively, myogenic conversion of nonmesenchymal cells might be dependent on their continued capacity to differentiate, i.e., only those cells would be able to respond in which genes required to regulate and express differentiation (including the expression of "ubiquitous" factors) are active. The latter hypothesis is largely substantiated by fusion experiments of a mouse muscle cell line with a number of human nonmuscle cells including normal keratinocytes and Hela cells (Blau et al., 1985). While in all heterokaryons with normal cells human muscle genes were activated heterokaryons with HeLa cells failed to express the human muscle markers analyzed. Accordingly, HeLa cells that are unable to differentiate in vitro or in vivo (our own unpublished observations) could also not be induced to express myogenic differentiation products after transfection with the MyoD1 gene (Weintraub et al., 1989). Thus, differentiation-related genes are possibly inactive in these cells and therefore not accessible for cooperation with the MyoD1 gene. However, these genes could obviously be reactivated after treatment with 5-azacytidine, a drug thought to reduce gene methylation (Blau et al., 1985).

Our interest is focused on human skin keratinocytes and respective cell lines that have maintained a high potential for epidermal differentiation. These cells provide excellent models to study the degree to which the authentic phenotype is maintained since, as stated above, epidermal differentiation is well characterized and a number of markers are available. We recently described the establishment of a spontaneously immortalized cell line (HaCaT) from human skin keratinocytes that is nontumorigenic and has largely retained the normal epidermal phenotype (Boukamp et al., 1988; Ryle et al., 1989). Manipulations, potentially able to alter epidermal differentiation, such as transfection and expression of the c-Ha-ras oncogene or malignant conversion, did not interfere substantially with its differentiation capacity (Boukamp et al., 1990*b*; Breitkreutz et al., 1991). This suggests that epidermal differentiation is a stable trait of these cells.

The HaCaT cells were used as a model of differentiating human keratinocytes to ask whether transfection of a single myogenic determination gene, the MyoD1 gene, can similarly induce myogenic differentiation in a cell of ectodermal linage as seen in heterokaryons. Hereby our intention was focused on analyzing a broad spectrum of epidermal differentiation markers since, to our knowledge, the interference of such foreign regulatory genes with the inherent epidermal differentiation program has not been studied extensively. Moreover, since epidermal differentiation, including morphogenesis (formation of a structured epidermis), remains incomplete under conventional culture conditions in vitro (Holbrook and Hennings, 1983; Breitkreutz et al., 1984), we additionally analyzed the transfectants under more physiological conditions in vivo (in surface transplants: Fusenig et al., 1983; Boukamp et al., 1985, 1990*a*) where normal keratinocytes and also the immortal HaCaT cells form a well structured and normally differentiated epidermis-like surface epithelium within one to two weeks (Boukamp et al., 1988, 1990*a*).Thus, the in vivo assay was particularly useful in allowing us to determine the influence of MyoD1 on tissue organization in addition to the expression of differentiation markers.

Materials and Methods

Scheme of evolution of cell variants and associated phenotypic changes:



Cell Cultures

The HaCaT cells were routinely grown in modified MEM (Fusenig and Worst, 1975) supplemented with 10% FCS and antibiotics at 37°C, 5% CO_2, 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 cover slips and either fixed when still subconfluent or after an additional 2–8 wk after confluency.

Transfection of Cells

HaCaT cells were transfected as described previously (Chen and Jones, 1990) using a calcium phosphate precipitate of 20 μg pEMSV scribe DNA or 20 μg pEMSV-MyoD1 DNA together with 2 μg pNeo3, a plasmid conferring resistance to the neomycin derivative G418. Selection in medium containing 400 μg G418/ml was started 48 h later. G418 resistant stable transfectants were ring isolated and maintained under continued selection.

5-aza-CdR Treatment

Cells (10^4/60-mm dish, Falcon Labware, Oxnard, CA) were treated with 5 μM 5-aza-2′-deoxycytidine (5-aza-CdR; Sigma Chemical Co., St. Louis, MO and München, Germany) 24 h after seeding. The drug was removed 24 h later by replacing it with fresh drug-free medium. Morphological changes were first apparent ~5 wk after treatment.

Southern Blot Analysis

DNA was extracted as described previously (Chen and Jones, 1990), digested with EcoRI (Boehringer Mannheim GmbH, Mannheim, Germany), run on a 1% agarose gel and transferred to Zetaprobe membrane (Cuno Inc., Meriden, CT) following the manufacturer's protocols. The mouse MyoD1 1.8-kb fragment was ^{32}P labeled using the procedure described by Feinberg and Vogelstein (1984). Hybridization was done using

the protocol of Church and Gilbert (1984). The blots were exposed to film (XAR; Eastman Kodak Co., Rochester, NY) for 1-7 d at −70°C.

Northern Blot Analysis

Total mRNA was isolated from cells lysed in 7.6 M guanidine hydrochloride (Cheley and Anderson, 1984), 20 μg of RNA samples were electrophoresed on 1% agarose, MOPS-2.2 M formaldehyde gels (Lehrach et al., 1977), transferred onto Zetaprobe membranes in 10× SSC (1.5 M sodium chloride, 0.15 M sodium citrate), UV cross-linked to the membrane, and hybridized as above.

Transplantation

Organotypic cultures were prepared as described previously (Boukamp et al., 1990*a*). Briefly, collagen gels (4 mg/ml) of rat tail collagen (type I) were mounted in Combi-ring dishes (Renner KG, Dannstadt, Germany) (Noser and Limat, 1987) and 2×10^5 cells were seeded onto the collagen surface. After ~17 h the medium covering the cells was drained and the collagen chamber was covered with a hat-like transplantation chamber (Renner KG). The whole unit was then transplanted onto the muscle fascia of the back of nude mice, as described (Boukamp et al., 1985, 1990*a*). Transplants were excised "en bloc" at days 4, 8, 12, 17, and 27 and either fixed in buffered formalin for histology or embedded in Tissue Tec (Lab-Tec. Div., Miles Laboratories Inc., Naperville, IL) and snap frozen in liquid nitrogen precooled isopentane and stored in liquid nitrogen for cryostat sections.

Indirect Immunofluorescence

Cells grown on glass coverslips were washed in PBS (3 × 5 min) before being fixed by serial immersion (5 min each) in 50, 100, and 50% acetone. After air drying the cells were rehydrated (10 min in PBS) and incubated with antibody solutions for 45 min in a moist chamber at room temperature. Antibodies used were: mouse mAbs against the differentiation-specific keratins K1 and K10 (Renner KG), the basal keratin 14 (kindly provided by Dr. E. Leigh); "simple epithelia" keratins K7, K8, K18, and K19 (Amersham Corp., Braunschweig, Germany); myosin (MF-20, Bader et al., 1982; a gift from Dr. D. Fishman); desmin (Dianova, Hamburg, Germany); the desmosomal proteins desmoplakin, plakoglobin, and desmoglein (Progen, Heidelberg, Germany); and cell surface glycoproteins GP90 and GP130 (kindly provided by Dr. E. Klein). A polyclonal rabbit antibody against MyoD1 was generously supplied by Dr. A. Lassar. For staining of the latter three the cells were fixed with 3.7% formaldehyde (in PBS) for 20 min followed by 10 min in −20°C methanol. The coverslips were washed in PBS (3 × 5 min) and incubated for an additional 45 min with an anti-mouse, anti-rabbit, or anti-guinea pig gamma globulin coupled to fluorescein-isothiocyanate (FITC) (Dianova, Hamburg, Germany), respectively. After another wash of 3 × 5 min, and a final rinse in 10 mM Tris-HCl buffer (pH 8.5), the coverslips were mounted onto slides with Aqua mount (Lerner Laboratories, New Haven, CT) and viewed under an inverted microscope (model IM 35; Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics.

For double immunofluorescence, a guinea pig anti-pankeratin (kindly provided by Dr. G. Bruder, Progen, Heidelberg, Germany) and a mouse mAb against vimentin (Progen) were used. The first antibodies were incubated overnight at room temperature, followed by a 1-h incubation with guinea pig anti-biotin and a 1-h incubation with a mixture of Texas red coupled to streptavidine and anti-mouse FITC. Similarly, 5-7-μm air-dried sections were rehydrated and the unfixed sections were treated as described above using a sequence specific antibody against K1, raised in guinea pig (Roop et al., 1984; kindly provided by Dr. D. Roop, Baylor College of Medicine, Houston, TX), a rabbit antisera against human involucrin (Watt, 1984; a gift from Dr. F. Watt), and a mAb against filaggrin (Paesel, Frankfurt, Germany).

Results

Stable Transfection of HaCaT Cells with Mouse MyoD1

The human epidermal HaCaT cells were transfected with a plasmid containing the mouse MyoD1 gene, driven by the Moloney sarcoma virus promoter, and the neomycin resistance gene to determine the influence of MyoD1 on the epidermal phenotype. Cultures transfected with the plasmid without the MyoD1 insert were used as controls. Herein, the differentiation behavior of a clone transfected with the plasmid containing the MyoD1 gene insert (clone-13) and a control clone (Vec-2; missing the MyoD1 insert) are mostly described in detail but all data were confirmed with a second independent set of clones (clone-6 and Vec-1).

Southern analyses were performed to confirm stable integration of the MyoD1 gene (Fig. 1 *A*). DNA extracted from HaCaT-, Vec-2-, and MyoD1-transfected clone-6 and clone-13 cells was digested with EcoRI to release the 1.8-kb MyoD1 cDNA insert. The 1.8-kb signal was absent in the parental HaCaT and Vec-2 cells but was present in clone-6 and clone-13 cells. Several high molecular weight bands were additionally visible for clone-13. These are most likely because of rearrangements that occurred during integration of the DNA into the host genome. In addition, a 6.8-kb fragment was present in all cells, representing the human homolog (Myf3; Braun et al., 1990) of the mouse MyoD1 gene that cohybridized under the hybridization stringencies used.

Expression of the MyoD1 Gene in the Clone-13 Cells

The cells were grown to confluence and maintained for up to 8 wk after confluence to provide optimal conditions for myotube formation. RNA was isolated from these cells and analyzed by Northern blotting, which showed that the exogenous 2.4-kb MyoD1 mRNA was not expressed in HaCaT or Vec-2 but was strongly expressed in clone-6 and clone-13 cells (Fig. 1 *B*). The number of MyoD1 expressing cells was determined by immunofluorescence with an antibody against MyoD1. 50–70% of the clone-13 cells expressed the gene at the protein level (Fig. 1 *C*) while Vec-2 cells were completely negative (Fig. 1 *D*).

Influence of the MyoD1 Gene on Cell Phenotype In Vitro

All transfected clones largely resembled the polygonal, tightly packed parental HaCaT cells in confluent cultures (Fig. 2 *A*). Islands of cells with more elongated mesenchymal morphology developed in postconfluent cultures of clone-13 (Fig. 2 *B*) and large multinucleated cells which strongly resembled myotubes could be detected in dense cultures kept for more than 3 wk after confluence. No such cells were seen in cultures of HaCaT or Vec-2 cells.

Cultures were incubated with antibodies against the muscle contractile protein myosin, and two intermediate filament proteins, desmin and vimentin, found in muscle or mesenchymal cells, to determine whether MyoD1 was able to induce the expression of these proteins. Neither HaCaT nor Vec-2 cells expressed any of these markers. Clone-13 cells, on the other hand, expressed myosin (Fig. 2 *C*) and desmin (Fig. 2, *D* and *E*) in 1–10% of the cells. Expression was highest after 2–4 mo postconfluence and here it was found to be present in myotube-like (Fig. 2 *D*) as well as small epithelial-like cells (Fig. 2 *E*). This long interval for maximal myogenic differentiation clearly differed from that observed with other cell types. Vimentin, on the other hand, was regulated differently since this protein was expressed in ~40% of clone-13 cells immediately after plating (Fig. 2 *E*) and the expression level did not increase further in post-

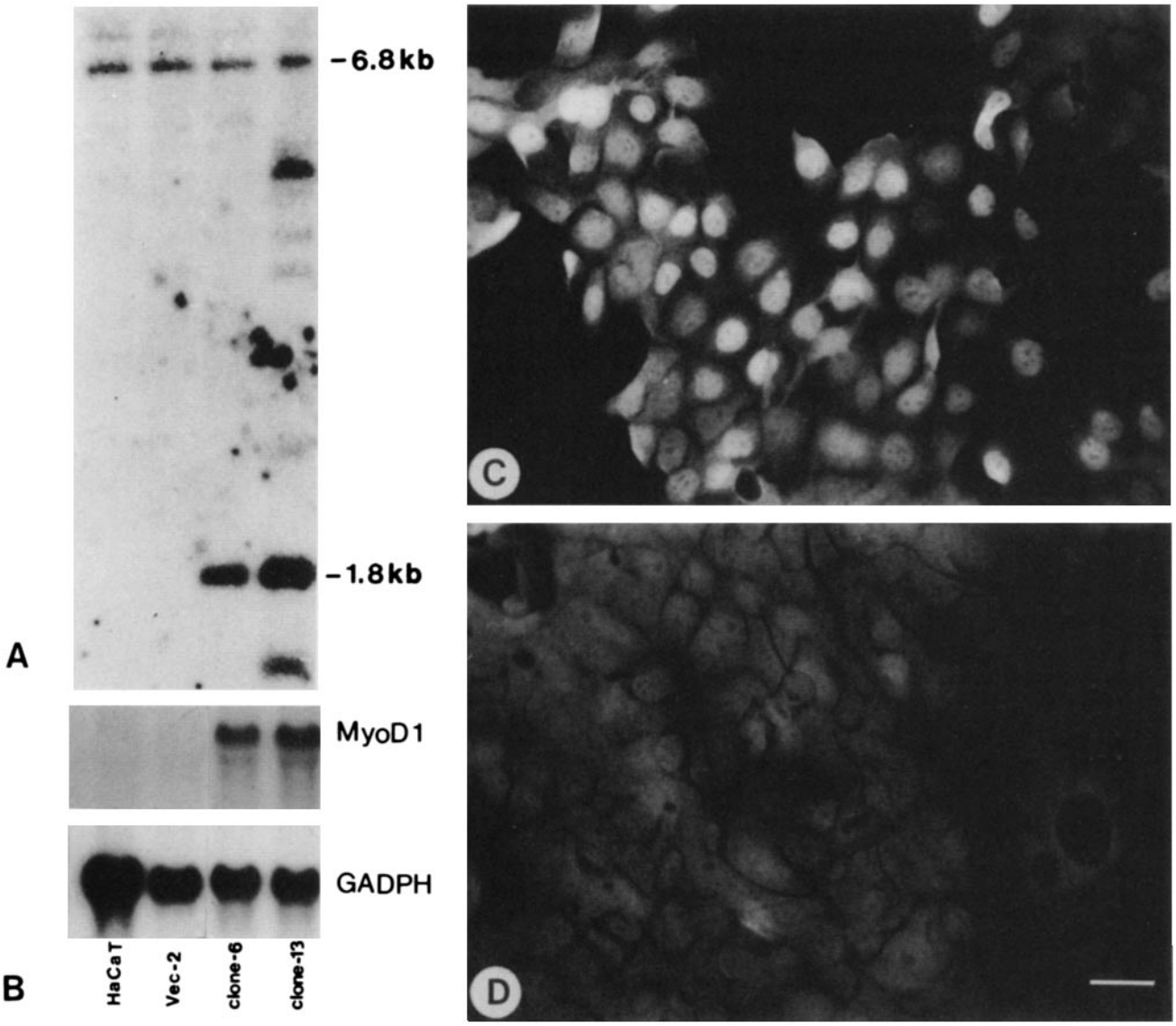


Figure 1. (*A*) Verification of integration of the mouse MyoD1 gene into the HaCaT genome. HaCaT, Vec-2 (control), and clone-6 and clone-13 (both MyoD1 containing) cell DNAs (10 μg) were digested to completion with EcoRI, electrophoresed on agarose gels, and transferred to nylon membrane. The Southern blot was hybridized with ^{32}P-labeled MyoD1 cDNA probe. The 1.8-kb band represents the MyoD1 insert while the 6.8-kb band represents the resident human homologue. (*B–D*) Expression of myogenic determination genes. (*B*) Northern blot analysis (20 μg) of total RNA isolated from postconfluent cultures (>6 wk, maximal myotube formation) of HaCaT, Vec-2, clone-6, and clone-13 cells after hybridization with ^{32}P-labeled MyoD1 probe. MyoD1 was not present in HaCaT and Vec-2 cells but expressed in clone-6 and clone-13 cells. (*Bottom panel*) Same blot hybridized with glyceraldehyde-3 phosphate-dehydrogenase (GADPH) indicating the concentrations of loaded RNA. (*C* and *D*) Immunofluorescence micrographs after labeling with an antiserum against MyoD1 of cultures from clone-13 (*C*) and Vec-2 cells (*D*). Bar, 25 μm.

confluent cultures. The epithelial nature of the vimentin-positive clone-13 cells was demonstrated by double labeling using a mouse monoclonal vimentin and a guinea pig polyclonal pankeratin antiserum. All vimentin-positive cells were also positive for keratin, but as expected, not all cells positive for keratin expressed vimentin (Fig. 2, *E* and *F*).

Epidermal Characteristics

Keratin profiles were analyzed using antibodies specific for the different keratins to determine whether the expression of mesenchymal/myogenic markers abrogated the production of proteins characteristically present in the epidermal HaCaT cells. Keratin 14, generally expressed in the basal layer of the epidermis and characteristic for squamous epithelia, was present in all HaCaT, Vec-2, and also clone-13 cells while K1 and K10, the differentiation-specific suprabasal keratins, were only seen in a few scattered cells in confluent cultures (Table I). In addition to the epidermal keratins, the HaCaT cells also express "simple epithelia" keratins (K7, K8, K18, and K19) (see also Boukamp et al., 1988; Ryle et al., 1989; Breitkreutz et al., 1991) characteristic for non-keratinizing epithelia (Moll et al., 1982*a*). This pattern remained unchanged for Vec-2 cells while a slight shift was

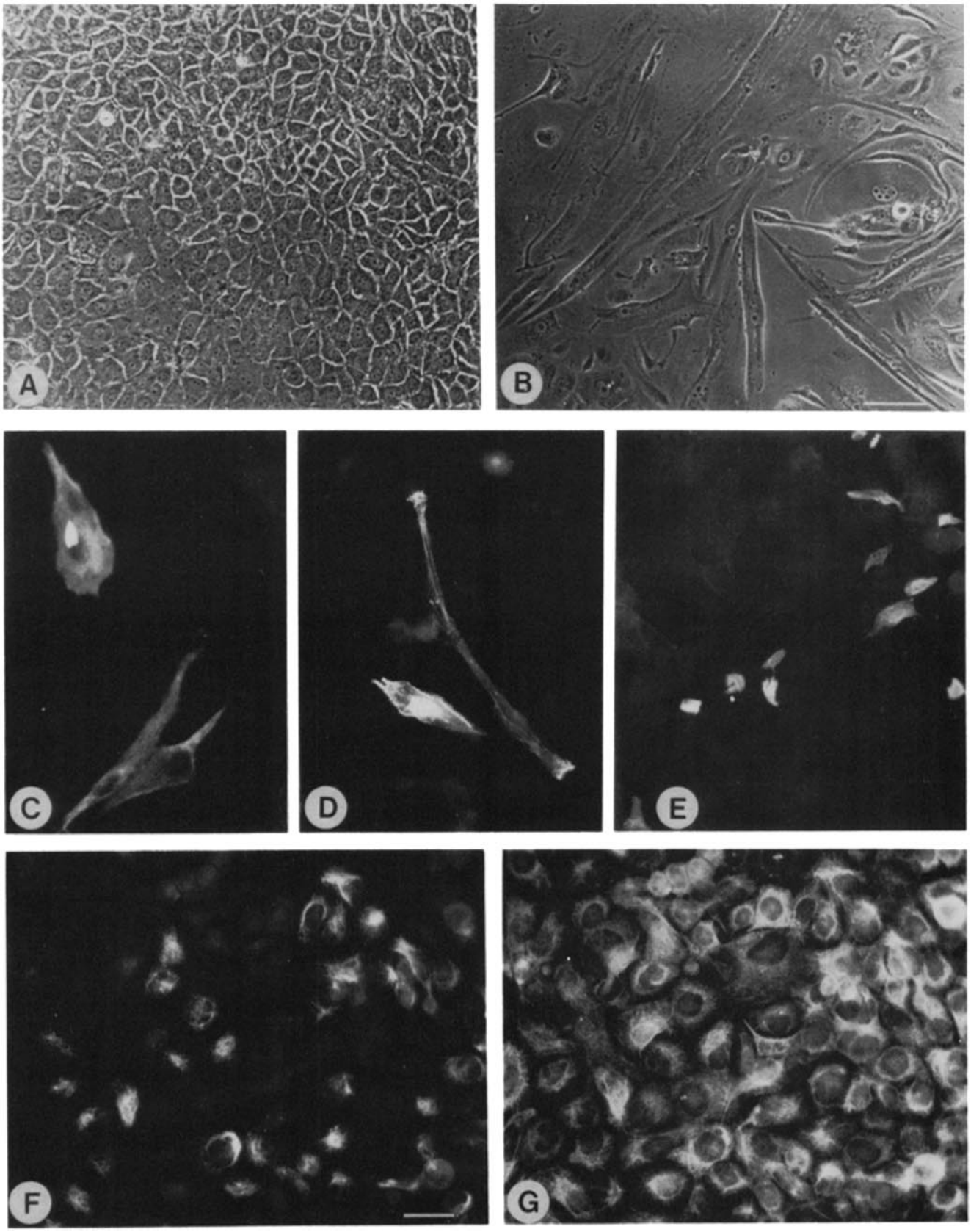


Figure 2. Phenotypic heterogeneity of the MyoD1 transfected clone-13 cells in culture. (*A* and *B*) Phase contrast micrographs of confluent cultures of clone-13 cells showing HaCaT-like morphology (*A*) and an island of elongated mesenchymal-like cells including multinucleated cells reminiscent of myotubes (*B*). (*C–F*) Immunofluorescence of myogenic differentiation products: Myosin (*C*); desmin (*D* and *E*), demonstrating that myotube- (*D*) as well as epithelial-like (*E*) cells express the myogenic marker. (*F* and *G*) Double fluorescence showing that vimentin (*F*) is expressed in ~40% of the cells that are all positive when a pankeratin antiserum (*G*) is used. The immunofluorescence was performed on fixed and permeabilized cells. Bars: (*A* and *B*) 100 μm; (*C–F*) 25 μm.

Table I. Expression of Keratins in HaCaT and Transfected Cells In Vitro

Keratin N°		HaCaT	Vec-2	Clone-13
14	Epidermal	+	+	+
1+10(11)	Keratins	(+)*	(+)*	(+)*
7		+	+	+
8	Simple Epithelia	+	+	−
18	Keratins	+	+	+
19		+	+	+

* Only single cells positive.

seen in cultures of clone-13 cells where K8 was no longer detected, suggesting a rather moderate influence on keratin expression by the transfected MyoD1 gene.

Differentiation Characteristics In Vivo

Complete epidermal differentiation is manifested by the formation of a multilayered well-organized tissue (stratified squamous epithelium) as well as the expression and correct spatial distribution of the different keratinization associated proteins. In general, this process remains incomplete under conventional culture conditions (Holbrook and Hennings, 1983; Breitkreutz et al., 1984) and thus, changes in morphogenesis are particularly difficult to assess in vitro. We therefore used a surface transplantation assay (Fusenig et al., 1983; Boukamp et al., 1990*a*) to analyze whether the capacity for tissue reconstitution of HaCaT cells under in vivo conditions was affected by expression of the MyoD1 gene.

When HaCaT cells were transplanted onto nude mice, they showed epidermal tissue architecture and the expression and correct spatial distribution of typical differentiation markers, thus largely resembling transplants of normal human keratinocytes (Boukamp et al., 1988, 1990*a*; Breitkreutz et al., 1991). The control cells (Vec-2) were not altered in their differentiation capacity (not shown) and clone-13 cells also formed a similarly well-structured epidermis-like surface epithelium with a distinct stratum granulosum and a parakeratotic, often massive stratum corneum within 3–4 wk after transplantation (Fig. 3 *A*). Typical epidermal differentiation markers were expressed and orderly distributed. Keratins K1 and K10 were present in all suprabasal layers (Fig. 3 *B*), filaggrin was expressed in the uppermost living cell layers just underneath the stratum corneum (Fig. 3 *C*), and an antibody to involucrin strongly stained the upper flattened living cell layers (Fig. 3 *D*).

Sections of the transplants were also labeled with antibodies against the myogenic differentiation products but were negative for desmin and myosin (not shown). Vimentin, on the other hand, was expressed in transplants of clone-13 cells (Fig. 3 *E*) while it was never seen in transplants of HaCaT or Vec-2 cells. Interestingly, the vimentin-positive cells were located in the basal layer of the epithelium. This restriction to the basal layer might indicate a controlled expression of vimentin in vivo. Thus, MyoD1 did not interfere with epidermal morphogenesis or the expression and correct spatial distribution of the differentiation markers despite the induced expression of vimentin in vivo.

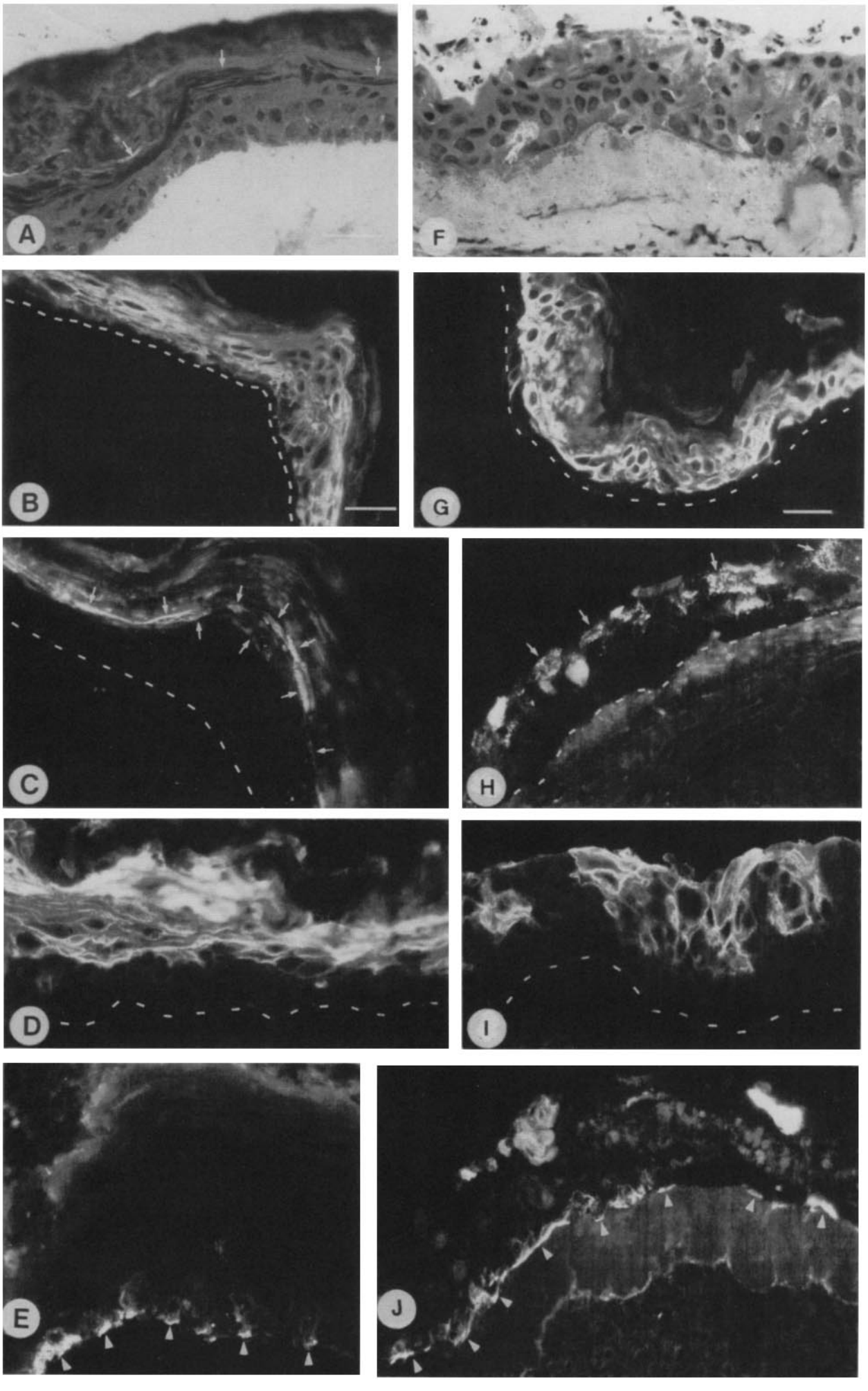
Derivation of Subclones from Clone-13 by 5-aza-2'-deoxycytidine Treatment

The myogenic conversion of MyoD1-transfected osteosarcoma cells can be potentiated by 5-aza-2'-deoxycytidine (5-aza-CdR) treatment (Chen and Jones, 1990). To determine the effect of additional 5-aza-CdR on the human epidermal cells we treated HaCaT, Vec-2, and the MyoD1 transfected clone-13 cells with the hypomethylating drug.

Treatment of control cells with 5-aza-CdR (HaCaT-aza and Vec-2-aza) did not lead to obvious phenotypic changes. The cells maintained their epithelial morphology in sparse and dense cultures. Furthermore, they neither expressed myogenic markers nor did they lose epidermal markers (not shown). In the 5-aza-CdR treated clone-13 cultures (clone-13aza) the number of MyoD1 expressing cells remained virtually unchanged, but the number of multinucleated cells resembling myotubes and mononucleated cells resembling spindle-shaped refractile myoblasts became more frequent in postconfluent cultures. Also, the number of myosin and desmin positive cells increased from ~5 to 15%. The keratin pattern, on the other hand, was not affected (data not shown).

Significant changes were, however, seen when the cells were transplanted onto nude mice. The control cells (HaCaT-aza and Vec-2aza) formed largely normal epidermis-like surface epithelia (not shown), whereas the clone-13aza cells formed a multilayered but morphologically disorganized and noncornified epithelium (Fig. 3 *F*). Surprisingly, when this epithelium was examined immunohistochemically, even markers of late stages in epidermal differentiation were expressed. Keratins K1 and K10 were present in nearly all suprabasal layers (Fig. 3 *G*) and filaggrin was localized predominantly in the uppermost cells (Fig. 3 *H*). Only involucrin occasionally showed an abnormal patchy distribution (Fig. 3 *I*). MyoD1 positive nuclei were irregularly distributed over the epithelium similarly as in transplants of clone-13 cells (data not shown). Thus, by 5-aza-CdR treat-

Figure 3. In vivo growth and differentiation behavior after transplantation as organotypic cultures (on a collagen gel) onto the muscle fascia of nude mice. The transplants were removed after 27 d, snap frozen, and cryostat sectioned. Transplants of (*A–E*) clone-13 cells and (*F–J*) clone-13aza cells. (*A*) Clone-13 cells formed a well-differentiated epidermis-like epithelium with distinct stratum granulosum (*arrows*) and massive stratum corneum. Comparable sections were labeled with antibodies against the differentiation-specific epidermal keratin K1, expressed in all subrabasal layers (*B*); filaggrin, the matrix component of the keratohyalin granules, labeling the stratum granulosum (*C*, see *arrows*) and involucrin, one of the precursor proteins of the cornified envelopes, giving rise to a membrane bound staining of the uppermost layers of the epithelium (*D*). Vimentin, the only mesenchymal/myogenic differentiation product present in vivo was restricted to the basal layer of the epithelium of clone-13 cells (*E*, see *arrowheads*). (*F–J*) Transplants of clone-13aza cells: histological section of the morphologically rather uniform epithelium with no indications of typical epidermal strata or cornification (*F*); similar section labeled with antisera against K1. As with clone-13 cell all suprabasal layers were positive (*G*); and the uppermost cells stained positive for filaggrin (*arrows*) (*H*). Involucrin occasionally showed an irregular distribution (*I*). Here too vimentin remained restricted to the basal layer of the epithelium (*J*, see *arrowheads*). Bar, 25 μm.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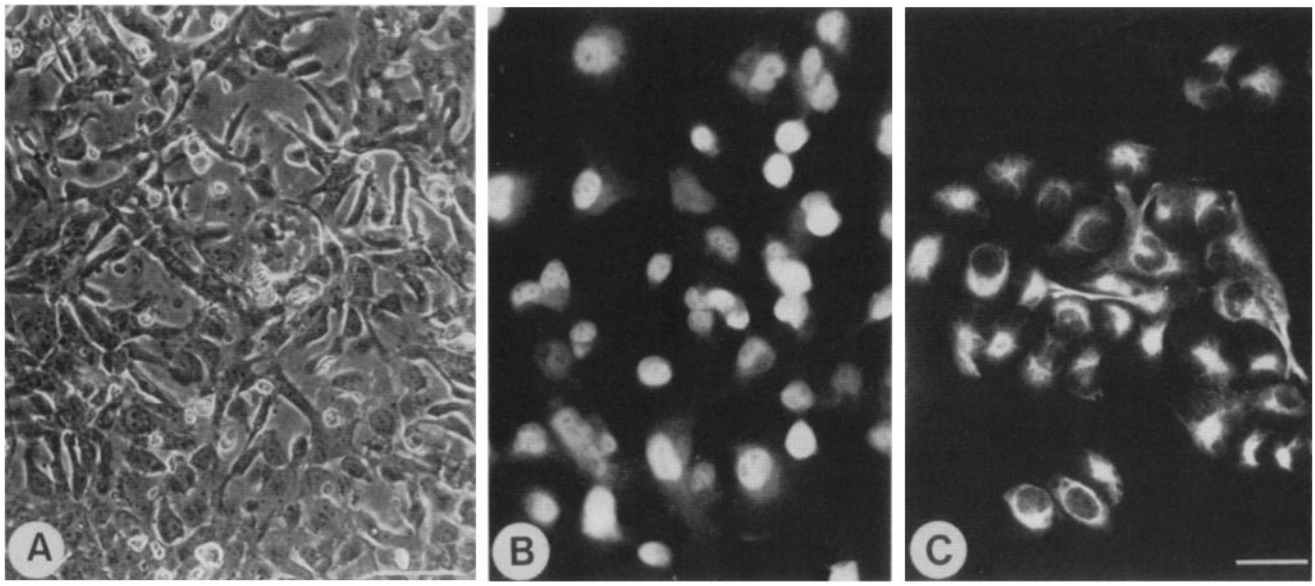

Figure 4. In vitro characteristics of the mesenchymal-like DTHMZ cells. (*A*) Phase contrast micrograph of the DTHMZ cells showing altered morphology. (*B* and *C*) Immunofluorescence micrographs of cultured DTHMZ cells labeled with antisera against MyoD1 (*B*) and vimentin (*C*). In both cases 100% of cells are positive. Bars: (*A*) 100 μm; (*B* and *C*) 25 μm.

ment of the MyoD1 transfected clone-13 cells, morphogenesis was disturbed while the expression of typical epidermal differentiation products remained unaffected. This showed that the processes of histo- and cytodifferentiation can be uncoupled and might therefore be regulated independently.

To determine whether the altered histogenesis was associated with the expression of myogenic differentiation products, sections were labeled with antibodies against myosin, desmin, and vimentin. Similar to the transplants of the untreated clone-13 cells myosin and desmin were not expressed in transplants of clone-13aza cells (not shown) and vimentin still remained restricted to cells of the basal cell layer (Fig. 3 *J*).

Table II. Comparison of Epidermal and Nonepidermal Differentiation Markers Between Clone-13aza and DTHMZ Cells

Differentiation markers		Clone-13aza	DTHMZ
Vimentin	myogenic	≈40%	100%
Myosin	differentiation	≈15%	≈30%
Desmin	markers	≈15%	≈30%
K14	epidermal	+	−
K1/10	keratins	(+)*	−
K8		−	−
K7	simple epithelia	+	+
K19	keratins	+	+
K18		+	(+)‡
Desmoplakin	desmosomal	+	−
Plakoglobin	proteins	+	−
Desmoglein		+	−
GP90	surface	+	−
GP130	glycoproteins	−	+

* Only single cells positive.
‡ Density dependent.

Mesenchymal Cell-like Subpopulation Differentially Trypsinized HaCaT-MyoD1 5-aza-CdR Treated Cells

During trypsinization it was observed that a fraction of cells detached from the plastic much more rapidly than the generally strongly adhesive HaCaT-like cells in clone-13aza cultures. We cannot exclude that a small number of these cells was already present in clone-13 cultures but they obviously could not be detected. Accordingly, cells with a comparable trypsinization behavior were never observed in cultures of HaCaT-aza or Vec-2aza cells nor during the numerous cloning experiments using HaCaT cells of different passage levels. Thus, by short term trypsin treatment of clone-13aza cultures a cell fraction was obtained with clearly altered adhesion properties.

Differentiation Properties In Vitro

These differentially trypsinized HaCaT-MyoD1 5-aza-CdR treated cells (DTHMZ)[1] cytologically no longer resembled HaCaT cells (Fig. 4 *A*). Moreover, 100% of the cells were now positive for MyoD1 (Fig. 4 *B*) and vimentin (Fig. 4 *C* and Table II). Also MyoD1 expression at the RNA level was increased in the DTHMZ compared to the clone-13 and clone-13aza cells (Fig. 5 *A*), thus correlating well with the immunofluorescence data. A human rhabdomyosarcoma cell line (*RD*) was used as a positive control.

The effect of MyoD1 expression of induction on other myogenic determination genes was measured next. A probe for Myf3, the human homolog of the mouse MyoD1 gene (Braun et al., 1990), was used to determine whether expression of the endogenous human gene was induced by the transfected mouse gene. Myf3 does not cross hybridize with MyoD1 under stringent hybridization conditions and this probe can

1. *Abbreviations used in this paper*: DTHMZ, differentially trypsinized HaCaT-MyoD1 5-aza-CdR treated cells; RD, human rhabdomyosarcoma cell line.

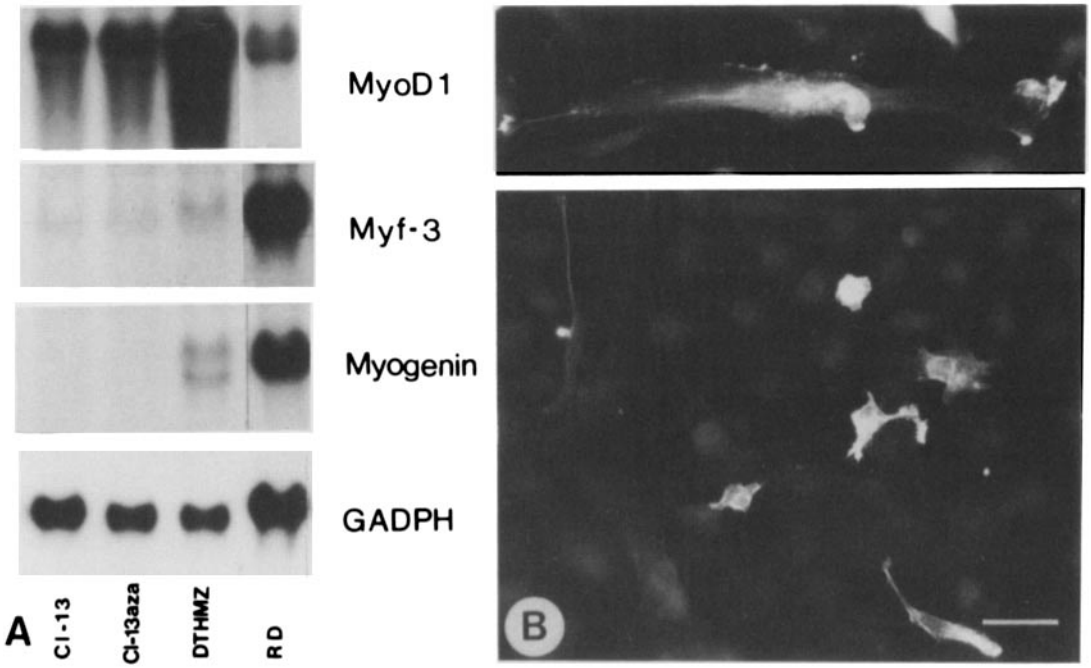

Figure 5. Myogenic properties of the DTHMZ cells. (*A*) Northern blot analysis of total RNA isolated from postconfluent cultures of clone-13, clone-13aza, DTHMZ, and a human rhabdomyosarcoma cell line (*RD*), used as a positive control for all probes. The blots were hybridized with ^{32}P-labeled MyoD1, Myf3 (the human homolog of MyoD1), myogenin, and GADPH probes; the latter as control for the concentrations of loaded RNA. MyoD1 was expressed in clone-13 and clone-13aza cells and strongly increased in DTHMZ cells. Myf3 could only be detected in DTHMZ cell while myogenin, clearly expressed in DTHMZ cells also showed a faint expression in clone-13aza cells. (*B*) Immunofluorescence micrographs of DTHMZ cultures where ~30% of the cells are labeled with an antibody against desmin; upper part shows a single large cell and lower part small myoblast-like cells that all stain positive for desmin. Bar, 25 μm.

therefore be used to assay for the expression of the endogenous human MyoD gene. The RD cells, used as a control, were positive for Myf3 expression as reported previously (Hiti et al., 1989) (Fig. 5 *A*). The Myf3 signal was absent in clone-13 and clone-13aza cells but was present in DTHMZ cells, demonstrating positive autoregulation by MyoD1.

We further assayed for the expression of myogenin, since previous studies have shown that this gene is associated with muscle cells and a positive regulatory loop has been described between MyoD1 and myogenin (Thayer et al., 1989; Chen and Jones, 1990). The control RD cells and the DTHMZ cells were clearly positive for myogenin expression and a faint signal was sometimes seen for the clone-13aza cells while the clone-13 cells were always negative (Fig. 5 *A*). Expression of two other myogenic determination genes, Myf5 (Braun et al., 1989) and herculin (Miner and Wold, 1990) was not detectable upon Northern blot analysis (data not shown).

To determine whether the increase in MyoD1 as well as expression of the other myogenic determination genes correlated with increased myogenic differentiation, cultures of DTHMZ cells were stained with myosin and desmin antibodies (Fig. 5 *B*). In both cases the number of positive myotube- and small epithelial-like cells had increased from ~15% for clone-13aza to ~30% for DTHMZ cells (see Table II) correlating with a comparable increase in MyoD1 positive cells.

On the other hand, the DTHMZ cells, unlike their progenitor clone-13aza cells, no longer expressed any of the epidermal keratins (basal K14 and suprabasal K1 and K10; Table II). Among the "simple epithelia" keratins K8 was absent, as already seen in cultures of clone-13aza cells, while K7 and K19 were maintained and gave rise to a filamentous pattern in most cells (not shown). K18, on the other hand, was expressed in a density dependent way. In subconfluent cultures 10–30% of the cells were positive, whereas at confluency all cells were negative, indicating a mode of regulation independent from that of K7 and K19.

Further significant changes were seen in the expression of cell surface markers including desmosomal proteins (plakoglobin, desmoglein, and desmoplakin) which are components of the typical epidermal junctions (desmosomes), being expressed in clone-13aza cells but not in the DTHMZ cells (Table II). The distribution of two cell surface proteins, GP90 and GP130, was also analyzed. Generally, the expression of the 90-kd glycoprotein is significantly increased in transformed human epithelial cell lines (Klein, C. E., B. Hartmann, L. Weber, and L. J. Old. 1989. *Immunobiology.* 178:36[Abstr.]) while the 130-kd glycoprotein is not expressed in normal epidermis but at high levels in capillary endothelial, smooth muscle and myoepithelial cells (Klein et al., 1988). While clone-13aza cells were strongly positive for GP90 and negative for GP130 as found for HaCaT cells (not shown) the reverse was seen for the DTHMZ cells, GP90 being negative and GP130 expressed in all cells (see Table II). Thus, the complete loss of epidermal markers including morphology, epidermal keratins, and desmosomal proteins), change in cell surface glycoprotein expression, expression of mesenchymal cytoskeletal proteins (vimentin

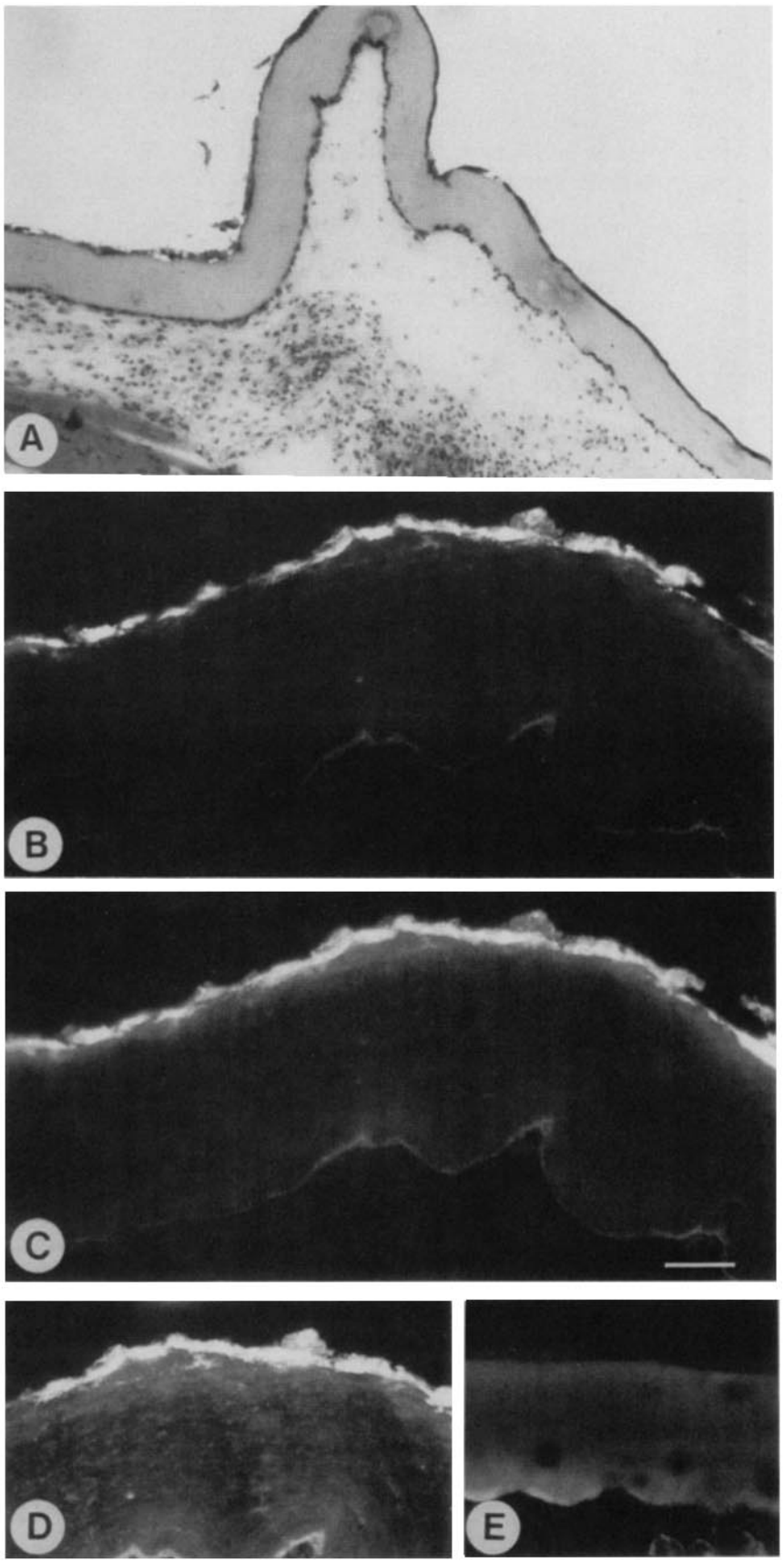

Figure 6. In vivo characteristics of the DTHMZ cells. (*A*) Histology of a 6-d-old transplant. Note the inability to stratify. (*B–E*) Immunofluorescence of the single-layered epithelium labeled with antisera against vimentin (*B*) and pankeratin (*C*) analyzed by double labeling show that all cells expressed both proteins; most cells are also positive for desmin (*D*) but negative for myosin (*E*). Bar, 25 μm.

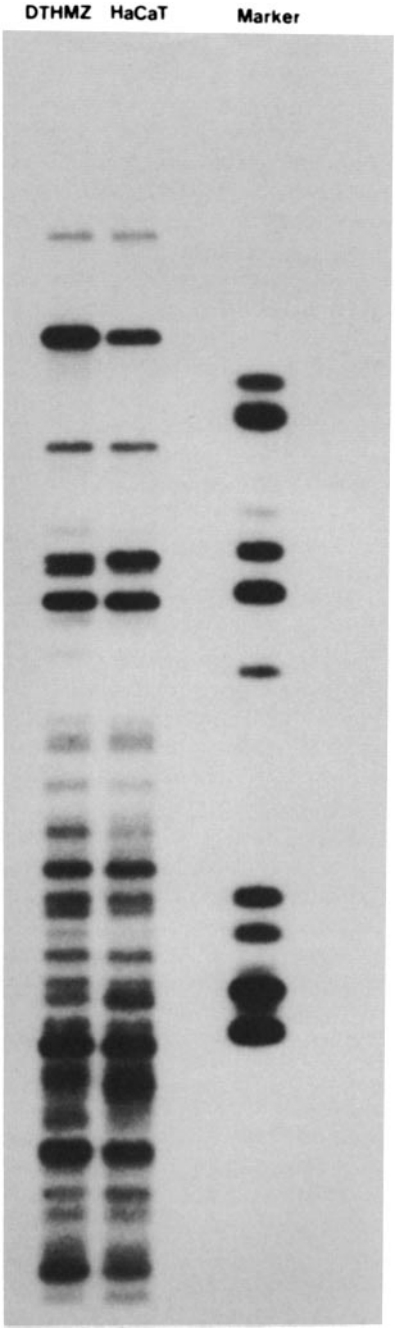

Figure 7. DNA fingerprint. Southern blot analysis of DNA from DTHMZ and HaCaT cells hybridized with the ^{32}P-labeled hypervariable minisatellite probe 33.15. The qualitatively identical pattern unequivocally demonstrates the HaCaT origin.

and desmin), the muscle protein myosin and additional myogenic determination genes (myf3 and myogenin) strongly argue for a shift from a clearly epidermal to a mesenchymal/myogenic phenotype rather than mere abrogation of epidermal differentiation.

Differentiation Characteristics of the DTHMZ Cells In Vivo

The DTHMZ cells did not stratify when transplanted onto nude mice but remained as a monolayer of flat elongated cells even after 27 d in vivo (Fig. 6 *A*). These cells reacted with antibodies against pankeratin and vimentin by double immunofluorescence (Fig. 6, *B* and *C*). Additionally, desmin was expressed (Fig. 6 *D*) while myosin could not be detected in these transplants (Fig. 6 *E*). Thus, also the in vivo growth behavior and expression pattern in the transplants support the hypothesis that complex changes were induced by the different treatments giving rise to "transdifferentiation" from the epidermal HaCaT cells to the mesenchymal/myogenic DTHMZ cells.

Proof for Descent of DTHMZ from HaCaT Cells

The DTHMZ cells were analyzed cytogenetically and by DNA fingerprinting to ensure that they were derived from the HaCaT cell line and to exclude cross-contamination with other mesenchymal cell lines. Firstly, the marker chromosomes defining the monoclonal origin of the epidermal HaCaT cells were still present in 100% of the DTHMZ cells (Pascheberg, U., personal communication; see Boukamp et al., 1988). Secondly, also the quantitatively identical DNA fingerprint pattern (Fig. 7) unequivocally demonstrated the common origin of the HaCaT and DTHMZ cells. Thus, by transfection of the MyoD1 gene into human keratinocytes followed by 5-aza-CdR treatment and further selection for decreased attachment to the substratum, a phenotypically highly divergent cell population had developed which could, however, clearly be identified as a HaCaT descendant.

Discussion

"Myogenic Conversion" by MyoD1 Transfection

Our studies demonstrate that the MyoD1 gene can induce the expression of myogenic differentiation products in the human epidermal HaCaT cells. The data are in line with findings by Blau et al. (1985) demonstrating that fusion of a mouse muscle cell line with a number of normal human cells, including keratinocytes, leads to the expression of human muscle markers. Similarly, Choi et al. (1990) could show that a number of different nontransformed cells, including retinal pigmented epithelial cells, can convert into striated mononucleated myoblasts and multinucleated myotubes after MyoD1 infection. Other epithelial cells such as HeLa, which have lost their differentiation capacity, appear to be refractory to myogenic conversion (Blau et al., 1985; Weintraub et al., 1989). These and our data strongly favor the hypothesis that cells which have maintained their potential to differentiate can be forced to myogenic differentiation by the MyoD1 gene. This might indicate that endogeneous differentiation related genes (gene products) are involved or are required to cooperate with MyoD1 to activate myogenic differentiation in nonmuscle cells. The level of myogenic conversion was low in the HaCaT cells compared with 10T1/2 and other cell lines. This might well be because of the fact that the epidermal cells are less predisposed towards myogenesis as discussed earlier for human osteoblastic TE85 cells (Chen and Jones, 1990). Similarly, for the human/mouse heterokaryons it was found that tissue derivation and possibly embryonic origin had marked effects on the kinetics and ultimate frequency of expression of muscle markers (Blau et al., 1985).

Two mechanisms have been proposed for the induction of

myogenic differentiation products, (*a*) direct activation by binding of MyoD1 to a consensus sequence (CANNTG) referred to as an E box (for review see Emerson, 1990), and (*b*) indirect activation for those genes lacking the consensus sequence. Depending on the respective activating mechanism one might expect a higher or lower level of expression of myogenic differentiation markers in nonmuscle cells. Myosin heavy chains, for example, have to be activated indirectly since they do not contain E boxes within their *cis*-regulatory regions (Bouvagnet et al., 1987). Expression of this gene was low (1–10%) in the clone-13 cells and this might indicate an inefficient transactivating mechanism in the nonmuscle cells. Vimentin, on the other hand, a cytoskeletal protein, not exclusively expressed in myogenic cells but associated with mesenchyme-derived cells, was expressed in ~40% of the MyoD1 transfected cells. This high percentage may be due to a direct regulation of vimentin by MyoD1 especially since vimentin obviously contains a CANNTG-like sequence in its 5′ regulatory region.

Vimentin is occasionally expressed in epithelial cells either in normal tissue during embryogenic development (Franke et al., 1982; Holthöfer et al., 1983; LeDouarin et al., 1984; Lehtonen et al., 1985; Paranko and Virtanen, 1986) or after oncogenic transformation (Franke et al., 1979; Fusenig et al., 1982; Azumi and Battifora, 1987; Raymond and Leong, 1989 and references therein; Domagala et al., 1990). Normal human keratinocytes apparently express vimentin at certain stages of growth in vitro (van Muijen et al., 1987*a*; Smola, H., G. Thiekötter, D. Breitkreutz, and N. E. Fusenig, manuscript in preparation) suggesting that the gene can be activated in epithelial cells by several factors. However, the HaCaT keratinocyte line has not been found to express vimentin under a variety of growth conditions in vitro and in vivo even after tumorigenic conversion following transfection with the Harvey-ras oncogene (Boukamp, P., unpublished observation). Thus, in the present case, vimentin induction was probably a direct consequence of MyoD1 transfection.

Stability of the Inherent Epidermal Phenotype

The HaCaT cells showed only minor changes in the expression of epidermal differentiation after MyoD1 transfection. The keratins, classified on the basis of molecular weights and charges (Moll et al., 1982*a*), show a characteristic pattern of expression for a particular cell type and tissue (Franke et al., 1984; Sun et al., 1984). The MyoD1 transfected clone-13 cells maintained all typical HaCaT keratins analyzed (epidermal keratins K1, K10, K14, and "simple epithelia" keratins K7, K18, and K19) (see also Ryle et al., 1989; Breitkreutz et al., 1991) with the exception of K8. The stability of the epidermal differentiation pattern in clone-13 cells was even more apparent under more physiological conditions in vivo. Despite the phenotypic changes seen in vitro, a well organized and differentiated epidermis-like epithelium, virtually indistinguishable from that of control (HaCaT and Vec-2) cells, was formed by the clone-13 cells. The expression and localization of the major differentiation products such as the keratins K1 and K10, filaggrin and involucrin were normal in concert with a regular tissue architecture. The only difference observed between clone-13 and the HaCaT or Vec-2 transplants was the additional expression of vimentin while myosin or desmin were not detected. However, unlike the situation in vitro where a high percentage of cells were vimentin-positive only a minor fraction of cells was positive for vimentin in transplants and these were generally restricted to the basal layer of the epithelium. These findings may indicate that coexpression of the differentiation specific keratins K1, K10, and vimentin is not compatible. Alternatively, vimentin could be masked (as known for K14) when cells move upward and differentiate, no longer being recognized by the antibody.

It also cannot be excluded that in vitro the growth and differentiation pathway is regulated differently from that in vivo since one important aspect is that the cells in vitro are not subject to the control of systemic factors or factors derived from the underlying mesenchyme. Our previous studies have clearly shown that complete epidermal differentiation including the status of tissue homeostasis is only obtained under mesenchymal influence (Boukamp et al., 1990*a*; Klingel et al., 1990; Fusenig et al., 1991; Smola, H., G. Thiekötter, D. Breitkreutz, and N. E. Fusenig, manuscript submitted for publication) and that in surface transplants the whole process only takes one to two weeks. Thus, it is very likely that in vivo (under the control of the mesenchyme) epidermal differentiation predominates while the myogenic differentiation pathway is not necessarily favored in this environment. Accordingly, the time span for a given cell in vivo (passing through the different cell layers) might be too short to express myogenic differentiation markers (others than vimentin) because of their long latency period already observed in vitro. Alternatively, myogenic differentiation might be completely downregulated under the in vivo conditions. Thus, the alterations after MyoD1 transfection seen under in vitro conditions obviously had no major effects on epidermal tissue morphogenesis and the correct spatial distribution of the epidermal differentiation markers in vivo.

5-Azacytidine Treatment Causes Loss of Tissue Morphogenesis

In a second step we tried to potentiate the MyoD1 activity in clone-13 cells by treatment with the hypomethylating agent 5-aza-CdR. This was done because the frequency of myogenic conversion in transfected osteosarcoma cells could be increased by such treatment (Chen and Jones, 1990). 5-Aza-CdR treatment caused a substantial change in the differentiation behavior of clone-13 cells in vivo. Although the cells were still able to stratify, a morphologically unstructured epithelium was formed with no indications of typical epidermal cell layers including cornification. Nevertheless, this epithelium still expressed the major differentiation products K1 and K10, filaggrin, and involucrin.

This morphological alteration was particularly striking since neither long-term passaging, tumorigenic conversion (Boukamp et al., 1990*b*), nor 5-aza-CdR treatment by itself (HaCaT-aza, Vec-2aza) interfered with the potential of HaCaT cells to develop a well-organized epidermis after transplantation. Thus, 5-aza-CdR treatment of MyoD1 transfectants (clone-13) resulted in a loss of morphogenic potential without blocking the expression of differentiation markers. This demonstrates that histo- and cytodifferentiation can be dissociated under certain conditions.

This dissociation can also be seen in tumors or tumor-derived cell lines since we had demonstrated earlier that a cell line established from a mouse skin carcinoma failed to

express K1 and K10 in transplants although these cells formed a morphologically keratinizing surface epithelium (Breitkreutz et al., 1986). Another example for dissociation of histo- and cytodifferentiation is the embryonic development of the epidermis. During embryogenesis the human epidermis develops from a two-layered epithelium expressing the "simple epithelia" keratins K8 and K18 (Moll et al., 1982*b*) and the cells start to stratify and express epidermal keratins as soon as a three-layered epithelium has formed (week 10). The switch from simple epithelia to epidermal keratins therefore appears at a "transitional stage" where epidermal morphogenesis is still incomplete. The epithelium obtained from the 5-aza-CdR treated clone-13 cells seems to resemble the transitional phase during embryogenic development.

Prendergast and Ziff (1991) have very recently shown that binding of the basic motif of *c-myc* to DNA is methylation sensitive in that binding is specific for unmethylated sites. MyoD1 is known to contain a region with high sequence homology to the c-*myc* family protein as well as a highly basic region (Lassar et al., 1989) so that binding of MyoD might be prevented by methylation in an analogous fashion to c-*myc*. Thus, methylation might be one possible mechanism to stabilize the final state of the well stratified adult epidermis. Since hypomethylation by itself was insufficient to destroy morphogenesis and control cells (HaCaT or Vec-2 cells treated with 5-aza-CdR) still formed a well-structured and differentiated epidermis-like epithelium, additional factors/gene products have to be required in early development that are obviously inactive in the adult epidermis. Whether MyoD- or generally Helix–Loop–Helix-like genes are involved will be studied.

Mesenchymal-like Subpopulation (DTHMZ): "Transdifferentiation"

The DTHMZ subpopulation isolated from clone-13aza cells, i.e., derived from a typical epidermal cell, had lost its epithelial morphology, changed the pattern of intermediate filament protein and cell surface glycoprotein expression, and was no longer able to stratify in vivo, associated with a lack of desmosomal proteins and expression of additional myogenic determination genes. However, its identity with the epidermal HaCaT cells was proven by cytogenetic analysis and DNA fingerprinting.

The fact that these cells were obtained by selection for reduced adhesion to plastic by short trypsinization strongly argues for changes in the expression of adhesion molecules. One possible candidate is gp90, a glycoprotein selectively expressed in adherently growing transformed cells (Klein, C. E., B. Hartmann, L. Weber, and L. J. Old. 1989. *Immunobiology.* 178:36[Abstr.]) and induced in suspension cultures of neuroblastoma cells selected for substrate adherence (Rettig et al., 1987). The marked decrease in adhesion by the DTHMZ cells was correlated with the loss of this protein. DTHMZ cells had also gained the expression of another surface glycoprotein, gp130, which is present at high levels in mesenchymal cells of various origins (Klein et al., 1988), but generally absent in normal epidermis and was not detectable in the parental clone-13aza cells. Together with the other findings, this switch in surface glycoproteins strongly argues for a "transdifferentiation" process from an epidermal to a mesenchymal/myogenic phenotype rather than mere down-regulation of some epidermal traits in the DTHMZ cells.

Support for this hypothesis was also evident from the Northern blot analysis. As mentioned earlier, Thayer et al. (1989) have shown that MyoD1 and myogenin appear to function in positive autoregulatory loops which include activation of the endogenous MyoD1 by the transfected MyoD1 c-DNA. This transactivation of the human homologue Myf3 (Braun et al., 1989) by the mouse MyoD1 as well as induction of myogenin in the DTHMZ cells is in good agreement with their improved myogenic potential. Expression of Myf5 and herculin, two other myogenic determination genes (Braun et al., 1989; Miner and Wold, 1990), was not detected in the DTHMZ cells. There is, however, evidence that MyoD1 and Myf5 are usually not coexpressed and that herculin (or Mrf4) is not expressed in most cultured myogenic cell lines (for review see Emerson, 1990). The expression of these two genes might be regulated differently as compared to the expression of MyoD1 (Thayer and Weintraub, 1990) and therefore the lack of expression in the DTHMZ cells could be because of more general phenomena rather than being specific for the epidermal origin of the cells.

The mesenchymal/myogenic nature of the DTHMZ cells was further supported by the transplantation studies. The DTHMZ cells were no longer able to stratify, but remained as a monolayer of flat elongated cells throughout the whole observation period unlike all other epithelial cells tested in our transplantation system. Even undifferentiated HeLa and hybrid cells derived from the fusion of HeLa with normal fibroblasts (Stanbridge et al., 1982) maintained the propensity to form a multilayered tissue (Bosch et al., 1990). Thus, the loss of stratification in the DTHMZ cells and the expression of vimentin and desmin in these transplants strongly argues for major phenotypic changes in this particular subfraction of cells. Because these cells are, however, true derivatives of the epidermal HaCaT cells, they might provide a valuable tool to gain a closer view of the regulatory mechanisms involved in stratification of epidermal cells and repression of stratification in mesenchymal-type cells.

The preserved expression of the "simple epithelia" keratins by the DTHMZ cells is more than a remnant of the epithelial origin since these keratins are often found to be present in nonepithelial cells (Knapp et al., 1989; and references therein). For example, K8 and K18 were shown to be coexpressed with desmin in developing myocardial cells of some vertebrate species (Kuruc and Franke, 1988) and keratin 19 was found to be present in smooth muscle cells in vitro and in vivo (Gown et al., 1988), indicating that coexpression of different types of intermediate filaments (keratins, vimentin, and desmin) is a more general phenomenon in nonepithelial cells; especially more than previously realized in fetal tissues (van Muijen et al., 1987*b*). Thus, the maintained expression of the keratins K7, K18, and K19 is clearly compatible with a fully differentiated mesenchymal/myogenic phenotype and consequently also compatible with the transdifferentiation process postulated for the DTHMZ cells.

In conclusion, our studies have shown that MyoD1 can induce the expression of muscle-specific gene products in the epidermal HaCaT cells confirming earlier findings that MyoD1 can act as a master switch gene in differentiating nonmyogenic cells. However, MyoD1 by itself did not significantly alter the epidermal differentiation characteristics

in vitro nor did it interfere with epidermal morphogenesis in vivo. By additional 5-aza-CdR treatment morphogenesis (histodifferentiation) and the expression of specific epidermal differentiation products (cytodifferentiation) could be dissociated suggesting at least two independent modes of regulation. After both MyoD1 transfection and 5-aza-CdR treatment and selection for reduced adhesion to the plastic substratum, a subpopulation was derived which had lost the epidermal characteristics and had acquired a mesenchymal-cell phenotype. Thus, these studies (*a*) underline the stability of the inherent epidermal phenotype and (*b*) also demonstrate processes leading to transdifferentiation in somatic cells (derived from adult tissue) in culture.

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