

# Normal Keratinization in a Spontaneously Immortalized Aneuploid Human Keratinocyte Cell Line

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**Abstract.** In contrast to mouse epidermal cells, human skin keratinocytes are rather resistant to transformation in vitro. Immortalization has been achieved by SV40 but has resulted in cell lines with altered differentiation. We have established a spontaneously transformed human epithelial cell line from adult skin, which maintains full epidermal differentiation capacity. This HaCaT cell line is obviously immortal (>140 passages), has a transformed phenotype in vitro (clonogenic on plastic and in agar) but remains nontumorigenic. Despite the altered and unlimited growth potential, HaCaT cells, similar to normal keratinocytes, reform an orderly structured and differentiated epidermal tissue when transplanted onto nude mice. Differentiation-specific keratins (Nos. 1 and 10) and other markers (involucrin and filaggrin) are expressed and regularly located. Thus, HaCaT is the first permanent epithelial cell line from adult human skin that ex-

hibits normal differentiation and provides a promising tool for studying regulation of keratinization in human cells. On karyotyping this line is aneuploid (initially hypodiploid) with unique stable marker chromosomes indicating monoclonal origin. The identity of the HaCaT line with the tissue of origin was proven by DNA fingerprinting using hypervariable minisatellite probes. This is the first demonstration that the DNA fingerprint pattern is unaffected by long-term cultivation, transformation, and multiple chromosomal alterations, thereby offering a unique possibility for unequivocal identification of human cell lines.

The characteristics of the HaCaT cell line clearly document that spontaneous transformation of human adult keratinocytes can occur in vitro and is associated with sequential chromosomal alterations, though not obligatorily linked to major defects in differentiation.

CELLS from several normal tissues of rodent origin can be malignantly transformed in vitro by various agents and can also undergo spontaneous neoplastic conversion after variable periods in culture (Sanford and Evans, 1982). Human cells have been found to be rather resistant in this respect (DiPaolo, 1983; for further references see Barrett and Tennant, 1985). The differences between human and rodent cells in their sensitivity to transforming agents in vitro have been attributed to several discriminating species-related factors such as natural life span, degree of inbreeding, and genetic stability. Since the incidence of neoplastic transformation in different species appears to correlate with the frequency of spontaneous chromosomal aberrations, the low incidence of complete transformation of human cells in culture has been mainly ascribed to the higher stability of the human genome (Sager et al., 1983; Kano and Little, 1985).

To date, transformation of human keratinocytes has only been achieved reproducibly by infection with simian virus-40 (SV40) or transfection with its isolated DNA (Steinberg and Defendi, 1979; Taylor-Papadimitriou et al., 1982; Banks-Schlegel and Howley, 1983; Brown and Parkinson,

1984; Rhim et al., 1985) resulting in potentially immortalized but nontumorigenic cell lines. These cells exhibited altered growth properties and substantial reduction of normal keratinization as well as partial reexpression of fetal characteristics (Banks-Schlegel and Howley, 1983; Steinberg and Defendi, 1983; Bernard et al., 1985). From these findings, as well as those from skin carcinoma cell lines (Rheinwald and Beckett, 1980; Tilgen et al., 1983; Boukamp et al., 1985) and from human tracheal and skin epithelial cell lines malignantly transformed in vitro using viruses or viral genes (Rhim et al., 1985; Yoakum et al., 1985), it has been concluded that deficiencies in differentiation are invariably associated with transformation and/or malignancy.

In contrast, we have demonstrated with spontaneously transformed mouse cells that immortality as well as malignancy can be compatible with a largely preserved differentiation potential and sensitivity to appropriate environmental signals (Breitkreutz et al., 1986; Fusenig et al., 1985; Bohnert et al., 1986). To explore whether this is also the case for transformed human keratinocytes, and to exclude virus-related effects on differentiation, we attempted to promote

spontaneous transformation in human skin keratinocytes by a variety of different culture conditions. Spontaneous transformation in vitro, including malignant progression of human cells, has been observed only rarely, even when cells were derived from cancer-prone patients (Azzarone et al., 1976; Danes and Sutano, 1982; Mukherji et al., 1984; Thielmann et al., 1983; Revoltella et al., 1986). During preparation of this paper another case of spontaneous transformation of human keratinocytes has been published (Baden et al., 1987a) using neonatal foreskin cells supposedly derived from a genetically affected individual.

Various methods for the propagation of human epidermal cells in vitro have been reported in recent years, mainly using irradiated 3T3 cells as feeder layers (Rheinwald and Green, 1975) or other modified culture conditions (e.g., Eisinger et al., 1979; Peehl and Ham, 1980; for review see Fusenig, 1986). The forced stimulation of proliferation achieved by these conditions and the presence of aneuploid transformed heterologous cells might not be optimal for transformation studies. Our experiments with mouse keratinocyte cultures have shown that spontaneous transformation to immortality and finally malignancy was best and most reproducibly achieved under conditions where cells could be successfully maintained in primary culture for long-term periods (Fusenig et al., 1982, 1985).

Using this approach we describe herein the spontaneous development of a cell line (HaCaT) from a long-term primary culture of human adult skin keratinocytes. These cells can be considered immortal (>140 passages), reveal a heteroploid stemline with specific stable marker chromosomes, but are not tumorigenic. Even after multiple passages HaCaT cells retain a remarkable capacity for normal differentiation and thus offer a suitable and stable model for keratinization studies. Moreover, this line could be reproducibly transfected with the activated human Ha-ras oncogene. Selected clones gave rise to highly differentiated benign epidermal cysts and/or squamous cell carcinomas in nude mice (Fusenig et al., 1987; Boukamp, P., D. Breitkreutz, E. Stanbridge, P. Cerutti, and N. E. Fusenig, manuscript in preparation).

## Materials and Methods

### Cell Isolation and Cultivation

Full thickness adult human body skin was obtained by surgical excision, in the case of HaCaT from the distant periphery of a melanoma located on the upper half of the back (not extensively sun-exposed) of a 62-yr-old male patient. The histology of the epidermis from the skin specimen obtained in a second, "safety" operation at the primary melanoma site showed no apparent anomalies. For cell isolation, skin was freed from fat and as much dermis as possible, cut into 1 cm<sup>2</sup> pieces, and placed on a 0.2% trypsin solution (1:250; Boehringer Mannheim, Mannheim, Federal Republic of Germany) in PBS without Mg<sup>2+</sup> and Ca<sup>2+</sup> at 4°C for 24–72 h depending on the thickness of the skin. Epidermis and dermis were then separated and cells isolated from both parts since the split level varied with different specimens (never located only at the basement membrane). Both cell fractions released from epidermis and dermis were pooled in complete culture medium of 4× MEM (a modified MEM, containing a fourfold concentration of aminoacids and vitamins, plus 15% heat-inactivated FCS and antibiotics; Fusenig and Worst, 1975), filtered through nylon gauze (100-μm mesh width), centrifuged, resuspended in complete culture medium, counted (Coulter counter; Coulter Electronics, Krefeld, FRG) and seeded at a density of 1.5 × 10<sup>6</sup> cells per 30-mm plastic petri dish (Falcon Labware, Oxnard CA) in medium containing ~1.4 mM Ca<sup>2+</sup> (high Ca<sup>2+</sup> 4× MEM). When islands had formed (by 5 d after plating) the Ca<sup>2+</sup> concentration was reduced to ~0.2 mM (low Ca<sup>2+</sup> 4× MEM) by using Ca<sup>2+</sup>-free 4× MEM, 5% FCS,

and 5% chelex-treated (Ca<sup>2+</sup> free) FCS according to Hennings et al. (1980). Ca<sup>2+</sup> concentrations were determined by atomic absorption. All media contained antibiotics (penicillin, 100 U/ml; streptomycin, 50 μg/ml). Cells were routinely cultured at 37 or 38.5°C in gassed (95% air and 5% CO<sub>2</sub>) humidified incubators. Subcultures were obtained by disaggregating the cells with 0.1% trypsin/EDTA solution (final concentration) in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS and replating at high cell density (1:2). Later passage cells were split at a ratio of 1:5 and eventually 1:10–1:20.

### DNA Fingerprinting

DNA was isolated from tissue, cultured cells, or peripheral blood by standard techniques. Digestion with Hinf I (Bethesda Research Laboratories, Gaithersburg, MD), agarose gel electrophoresis, Southern blotting, and hybridization with the hypervariable minisatellite probes 33.15 or 33.6 were performed as described by Jeffreys et al. (1985).

### Growth Behavior In Vitro

**Colony-forming Efficiency on Plastic.** 10<sup>3</sup> cells were seeded per 60 mm dish (five dishes per experiment). After 2–4 wk incubation at 37°C or 38.5°C, respectively, the cultures were fixed in formalin (3.7% in PBS), stained with hematoxylin and Rhodamine, and the colonies counted macroscopically. Cloning efficiency was expressed as percentage of counted colonies to the total number of plated cells.

**Population Doubling Time.** Growth curves were plotted over a 7-d period. 1 × 10<sup>5</sup> of normal keratinocytes or 5 × 10<sup>4</sup> HaCaT cells were plated per 35-mm dish and three dishes were counted at daily intervals.

### Cytogenetic Analysis

Semiconfluent cultures were treated for 2 h at 37°C with medium containing 0.04 to 0.08 μg/ml colcemid. The cells were detached by subsequent treatment with 0.1% EDTA (5–8 min) and EDTA/trypsin (1/1, 0.2% each; 5–8 min), centrifuged, and the cell pellet resuspended in a hypotonic solution of 75 mM KCl. After incubation for 10–15 min at room temperature, cells were fixed by three changes of methanol/acetic acid (3:1), spread on glass slides, and G-banding carried out after 2–3 wk as described (Boukamp et al., 1982). Usually 100 metaphases were analyzed microscopically and at least 20 karyograms constructed.

### Growth and Differentiation Behavior In Vivo

**Tumorigenicity Test.** Tumor formation was assayed after subcutaneous injection of up to 5 × 10<sup>6</sup> cells in 100 μl culture medium into the interscapular region of 4–6-wk-old nude mice (BALB/c nu/nu backcrosses) over an observation period of at least 6 mo.

**Transplantation of Cell Suspensions onto the Muscle Fascia.** 1 × 10<sup>6</sup> normal keratinocytes or 5 × 10<sup>5</sup> HaCaT cells in 200 μl complete culture medium were transplanted onto the muscle fascia at the interscapular region of 6-wk-old nude mice as described (Boukamp et al., 1985). Briefly, a hat-like silicon transplantation chamber (Renner GmbH, Darmstadt, FRG) was inserted by an incision of the back skin and kept in place by tightly fixing the wound margins of the skin with wound clips. The cell suspension was seeded to the graft site by direct injection through the chamber. For histology or cryostat sections transplants were excised at weekly intervals and either fixed in buffered formalin or embedded in Tissue Tec (Lab Tec Prods, Naperville, IL) and snap frozen in liquid nitrogen, respectively.

### Transplantation on Collagen Gels

Alternatively intact cultures grown on collagen substratum were transplanted essentially as described for mouse keratinocytes by Worst et al. (1982). Collagen type I from mouse tail tendon (lyophilized acetic acid extracts adjusted to 3 mg/ml) was gelled (300 μl) in the silicone culture chambers (Renner GmbH) by exposure to ammonia vapor (1.9% ammonia, 1 h) as described previously (Fusenig et al., 1982; Bohnert et al., 1986) and fixed with glutaraldehyde (4% for 1 h). After extensive washes in PBS and complete culture medium, 2 × 10<sup>5</sup> cells in 200 μl medium were seeded. The chambers were placed in Stanzen petri dishes (Greiner uS, Nürtingen, FRG) providing free medium access from below, and incubated at 37°C in a humidified gassed atmosphere. After 24 h cultures were rinsed and the medium covering the cells was drained. The chamber was covered with the hatlike transplantation chamber and the complete unit was transplanted onto the muscle fascia as described above.

## Indirect Immunofluorescence

Cryostat sections of 5–7  $\mu\text{m}$  were air-dried and incubated with the antisera listed below for 45 min at room temperature, as described by Boukamp et al. (1985). The antiserum against human involucrin was raised in rabbits (Watt, 1984) and kindly provided by Dr. Fiona Watt (Kennedy Institute of Rheumatology [JCRF], London, United Kingdom). The rabbit antiserum against rat filaggrin (Scott and Harding, 1986), which cross-reacted with the human protein, was a gift from Dr. Ian Scott (JCRF, Unilever Research, Colworth Laboratory, Bedford, U.K.). The monoclonal antibody identifying basal cells (Pab 421; Leigh et al., 1985) was kindly provided by Dr. Birgit Lane (London, UK). A rabbit antibody directed against the COOH-terminal sequence of the acidic 58K mouse keratin (corresponding to human No. 10/11; Roop et al., 1984) cross-reacting with the human 57K suprabasal keratin was donated by Dr. Dennis Roop (National Cancer Institute, Bethesda, MD). After several washes in PBS sections were labeled with the respective second antibodies, all purchased from Miles-Yeda (Rehovot, Israel), for an additional 45 min, washed in PBS and Tris-HCl buffer (pH 8.5), embedded in Aqua mount (Lerner Laboratories, New Haven, CT), and viewed under a Zeiss inverted microscope (IM 35) equipped with epifluorescence optics.

## Protein Analysis

Transplants of HaCaT cells and normal human adult keratinocytes (both on glutaraldehyde-fixed collagen gels) were dissected and the epidermal tissue mechanically removed after incubation in 10 mM EDTA at 4°C for 3 h. Cytoskeletal proteins were prepared by sequential extraction using low and high salt buffers containing nonionic detergent (Triton X-100) at 4°C following our previous protocol (Breitkreutz et al., 1984, 1986). Briefly, all samples were homogenized at least twice in high salt buffer and cytoskeletons were thoroughly washed in low salt buffer and extracted in 1% SDS sample buffer (25 mM dithioerythritol, 0.5 mM  $\text{MgCl}_2$ , 20 mM Tris-HCl, pH 7.4). The samples were first homogenized on ice (glass-glass homogenizer, potter type), heated for 20 min at 90°C, homogenized once more, and after a 2-h extraction, cleared by centrifugation (40,000 g for 30 min at 22°C).

**Two-dimensional Gel Electrophoresis.** Samples dissolved in 1% SDS sample buffer were adjusted to 9.5 M urea and diluted with 2 vol sample dilution buffer (containing 9.5 M urea, 4% NP-40, 5% mercaptoethanol and 3% ampholines (pH 5–8; LKB Produkter AB, Bromma, Sweden) as described previously (Bowden et al., 1984). Separation in the first dimension was achieved by nonequilibrium pH gradient electrophoresis (NEpHGE) in tube gels using a wide ampholine range (pH 2–11) for 3,000 volthours (Vh). The gels were equilibrated in buffer containing 50 mM Tris-HCl, pH 6.8, 1% SDS, 1 mM EDTA, and 0.5% mercaptoethanol and separated in the second dimension by SDS-PAGE (7.5–17.5% polyacrylamide gradients, see Bowden et al., 1984).

## Results

### Development of the HaCaT Cell Line

Keratinocytes from full thickness adult human skin were generally isolated by separation of epidermis and dermis with trypsin at 4°C (Kitano and Endo, 1977) and plated at high cell density without a 3T3 feeder layer. In the case of the HaCaT cells, a histologically normal male skin specimen was obtained from the distant periphery of a melanoma (second excision). The cells were plated in high  $\text{Ca}^{2+}$  4 $\times$  MEM in 12 35-mm dishes with  $1.5 \times 10^6$  cells in each. Since long-term growth in primary culture (without passaging) was optimal in low  $\text{Ca}^{2+}$  medium (0.2 mM), probably due to a marked reduction in terminal differentiation, six cultures were shifted to low  $\text{Ca}^{2+}$  medium 5 d after plating. Moreover, elevation of culture temperature (e.g., 38.5°C) had previously been shown to increase proliferative activity and to prolong the average life span from 2 up to 3 mo. Thus, all dishes were further propagated at 38.5°C.

1. *Abbreviation used in this paper:* NEpHGE, nonequilibrium gradient electrophoresis.

In one culture grown in low  $\text{Ca}^{2+}$  4 $\times$  MEM at 38.5°C, a colony continued to proliferate beyond 3 mo and by 5 mo after plating had nearly covered the dish. Subculturing with routine methods (EDTA/trypsin) was unsuccessful, but cells, detached by vigorous pipetting, reattached after transfer to a new dish and grew to confluency within 4–6 wk. From this first subculture cells could subsequently be passaged by trypsinization but proliferation strictly depended on plating at high cell density (split ratio 1:2). By the fourth passage, cell density was no longer critical to maintenance of satisfactory proliferation. The resulting cell line was designated HaCaT to denote its origin from human adult skin keratinocytes propagated under low  $\text{Ca}^{2+}$  conditions and elevated temperature.

Morphologic differentiation of skin keratinocytes in culture, visible as stratification and squame formation, was usually most prominent in high  $\text{Ca}^{2+}$  medium with focal keratinization in subconfluent cultures. In low  $\text{Ca}^{2+}$  medium, cultures predominantly remained as monolayers with single cells (mostly cornified envelopes) shed into the medium as described earlier by Hawley-Nelson et al. (1980). Additionally, cell morphology changed from densely packed polygonal cells in high  $\text{Ca}^{2+}$  medium (Fig. 1 A) to a more heterogeneous pattern with elongated loosely arranged cells in low  $\text{Ca}^{2+}$  medium (Fig. 1 B).

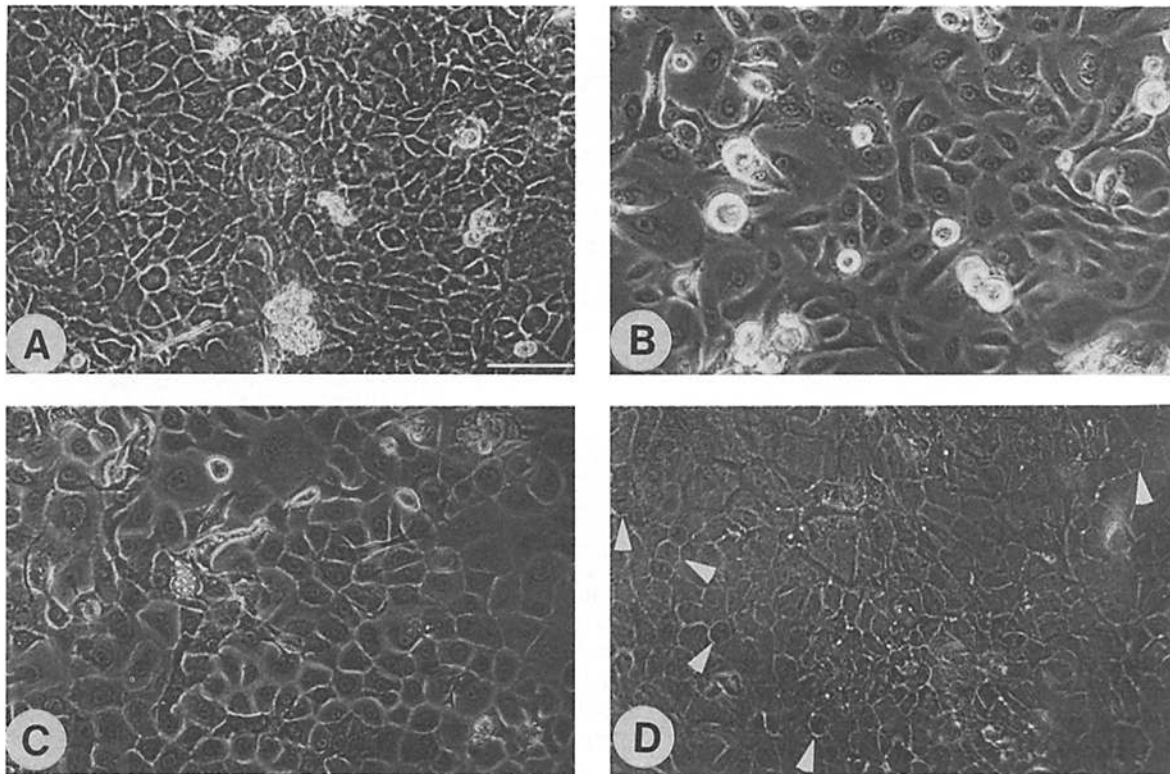
The HaCaT cells when maintained under the initial conditions (low  $\text{Ca}^{2+}$ , 38.5°C), displayed an intermediate phenotype of polygonal cells interspersed with giant often multinucleated cells (Fig. 1 C) and single cornified envelopes. When the calcium level was raised (to 1.4 mM) the cultures readily stratified and formed coherent horn sheets. In contrast to their untransformed counterparts, however, the cells continued to proliferate (Fig. 1 D) although at a lower rate. With subsequent passages stratification and horn sheet formation steadily decreased but could be reinduced in transplants in vivo (see below). The epithelial nature of the cells under both culture conditions was confirmed by immunofluorescence with antibodies against cytokeratin as well as by their keratin profiles on one- and two-dimensional polyacrylamide gels (data not shown). Vimentin expression could not be detected by either method.

### Altered Regulation of Proliferation

After prolonged cultivation HaCaT cells gradually escaped from environmentally modulated growth control in vitro and eventually became autonomous, i.e., capable of clonal growth under various culture conditions. During propagation for more than 140 passages (undergoing at least 600 population doublings) no obvious crisis was noticed.

The HaCaT cells did not show contamination with mycoplasmas when tested at regular intervals with cytochemical technique (Dapi) for demonstration of DNA in infected cells. Similarly, no expression of SV40 large T antigen could be detected by immunofluorescence and Southern blot analysis under low stringent conditions excluded the presence of human papilloma virus (HPV) sequences in the HaCaT cells (M. Dürst, personal communication).

When first tested (at passage 5) HaCaT cells already exhibited altered growth properties compared with normal keratinocytes and were able to grow at clonal densities ( $10^3$  cells per 60 mm dish, see Table I). However, at that early



**Figure 1.** Phase-contrast micrographs of normal human skin keratinocytes in culture (*A* and *B*) and early passage HaCaT cells (*C* and *D*). (*A*) Normal keratinocytes grown in high  $\text{Ca}^{2+}$   $4\times$  MEM (1.4 mM) and (*B*) in low  $\text{Ca}^{2+}$   $4\times$  MEM (0.2 mM). (*C*) Early passage HaCaT cells grown in low  $\text{Ca}^{2+}$   $4\times$  MEM exhibit an altered phenotype due to a more densely packed monolayer interspersed with giant, often multinucleated, cells. (*D*) After the shift to high  $\text{Ca}^{2+}$   $4\times$  MEM HaCaT cultures are covered with coherent horn sheets but mitoses (*arrowheads*) remain frequent. (Bar, 50  $\mu\text{m}$ ).

stage the cells were still very sensitive to modifications of the original culture conditions (0.2 mM  $\text{Ca}^{2+}$ , 38.5°C). Reduction of the culture temperature (to 37°C) or elevation of the  $\text{Ca}^{2+}$  level (to 1.4 mM  $\text{Ca}^{2+}$ ) resulted in a drastic decrease in cloning efficiency and a prolonged population doubling time. Around the tenth passage cells developed complete independence of both  $\text{Ca}^{2+}$  concentration and temperature. With further passages, however, cloning efficiency and growth rate did not change significantly.

The ability to grow in soft agar appeared to be a relatively late event and was first noticed at passage 18 although with a low efficiency that subsequently remained unchanged. Agar colonies were viable as judged by staining and replating of selected clones.

Although the HaCaT cell line exhibited a transformed phenotype *in vitro*, was clonogenic on solid and in semisolid substrata, and presumably immortalized, cells were neither tumorigenic after subcutaneous injection (up to passage 100, so far tested) nor invasive in a more sensitive transplantation assay (see Boukamp et al., 1985). Small nodules that developed shortly after injection regressed within 3–6 wk and late recurrences were not seen within 12 mo of observation.

### Cytogenetic Characteristics

The chromosomal constitution of HaCaT cells was followed during propagation, starting with passage 2, when the cells had been in culture for a total of 8 mo. At this early stage distinct numerical and structural karyotypic alterations were

obvious (Table II). The majority of cells were hypodiploid with an average of 44 chromosomes resulting from full or partial monosomies of chromosomes involved in the formation of marker chromosomes. All metaphases had the XO sex chromosome constitution (lacking the Y chromosome) and were partially monosomic for the short arm of chromosome Nos. 3, 9, and 4 (later monosomy of whole No. 4) while trisomic for the long arm of No. 9 (due to i[9q]). Three individual marker chromosomes (M1, t(3q4q); M2, i(9q); and M3, del(4q28)) were present in 100% of the metaphases and clearly indicated the clonal origin of this line (Fig. 2 *A*). Moreover, these individual cytogenetic characteristics excluded cross-contamination with other human cell lines developed and/or used in our laboratory.

Starting at passage 5 a hypotetraploid stem line evolved with a range of 72–88 chromosomes (including duplication of the early markers) and a fourth marker chromosome (M4 [4p18q]) appeared, replacing M3 (Fig. 2 *B*). These cytogenetic alterations were apparently linked to changes in growth control, indicated by improved adaptation to growth under modified culture conditions. With further passages additional markers developed, mainly involving chromosome Nos. 1, 6, 15, 17, and 22. However, these late structural aberrations were found in only 15–50% of metaphases.

### Identity of HaCaT Cell and Donor DNA

To prove the origin of the HaCaT cell line from the original donor and to exclude cross-contamination with other human

**Table I. Adaptation of the HaCaT Cell Line to Autonomous Growth during In Vitro Propagation**

Culture passages	Culture temperature	Cloning efficiency on plastic	Population doubling time	Cloning efficiency in soft agar	Tumorigenicity
<i>n</i>	°C	%	<i>h</i>	%*	
5	38.5	7.5	26 (0.2 mM Ca <sup>++</sup> )		
	37	0.9	50	ND	—
7	38.5	ND	38	ND	—
	37		39		
11	37	7.8	ND	ND	ND
15	37	10.8	22	—	ND
18	37	ND	ND	0.24	—
29	37	14.2	ND	0.27	—
37	37	13.3	23	0.27	—
48	37	13.2	23	0.36	—
81	37	ND	21	ND	—

\* As tested in high Ca<sup>++</sup> 4× MEM (1.4 mM) unless specified. ND, not done.

cell lines (in addition to evidence from karyotyping), HaCaT cells at various passage levels were analyzed by DNA fingerprinting (Jeffreys et al., 1985). Using the hypervariable minisatellite probes 33.15 and 33.6, we demonstrated the identity of the DNA fingerprints obtained from the donor tissue with that of the HaCaT cells. Moreover, despite the documented drastic cytogenetic changes during prolonged cultivation of the HaCaT cell line, the highly characteristic DNA fingerprint pattern remained unchanged from passages 6–79 (Fig. 3). The results were absolutely reproducible and identical patterns were obtained in five independent analyses. For further confirmation, DNA fingerprints prepared from eight other cell lines (previously developed or grown in our laboratory, including HeLa) and two different patients' blood samples were all unique and showed no similarity to the pattern of the HaCaT cells (see Fig. 3).

**Growth and Differentiation Behavior In Vivo**

To date, complete differentiation of keratinocytes, including regular tissue organization and normal expression of differentiation products, has only been shown to occur under mesenchymal influence (Worst et al., 1982; Lavker and Sun, 1983; Breitzkreutz et al., 1984, 1986; Bohnert et al., 1986; Fusenig, 1986). Thus, HaCaT cells were further analyzed for their differentiation potential after injection into or transplantation onto athymic nude mice. When injected subcutane-

ously cells developed nodules that, with later passages, occasionally persisted over several weeks. Histological sections of these nodules revealed large encapsulated cysts often filled with horny squames, while the lining epithelium was rather thin or atrophic (data not shown).

Similar encapsulated cystic structures were formed when suspensions of HaCaT cells were transplanted onto the subdermal muscle fascia as described for human skin carcinoma cells (Boukamp et al., 1985). 1–2 wk after transplantation the lining epithelium of these cysts had developed a regular tissue architecture including stratum granulosum and corneum (Fig. 4 A). The living epithelium eventually degenerated, leaving behind a cyst filled with horny material. This growth and differentiation pattern, characteristic for all passage levels tested (up to 80), was very similar to that of transplanted normal human skin keratinocytes.

When transplanted as intact cultures growing on glutaraldehyde-fixed collagen gels, contact between keratinocytes and host tissue (supposedly leading to encapsulation of the epithelial cells) was prevented. Under these circumstances HaCaT cells reconstituted an almost perfect epidermis (Fig. 4 B) similar to that formed by normal adult keratinocytes (Fig. 4 C). However, normal tissue architecture was only observed after a delay of 1–2 wk in HaCaT transplants, indicating reduced sensitivity to environmental signals. With later passages the epithelium formed in vivo showed slight devia-

**Table II. Chromosomal Changes of HaCaT Cells during Adaptation to Autonomous Growth In Vitro**

Passage no.	Numerical distribution (percent of metaphases)			Marker chromosomes* (percent of metaphases)				
	Diploid (46)	Hypodiploid (38–45)	Hypotetraploid (72–88)	M1	M2	M3‡	M4‡	M5-M8§
2	10	90	0	100	100	100	0	0
5	0	67	33	100	100	77	23	5
11	0	58	42	100	100	70	30	8
17	0	0	100	100	100	0	100	25
33	0	0	100	100	100	0	100	100¶
50	0	0	100	100	100	0	96	100†

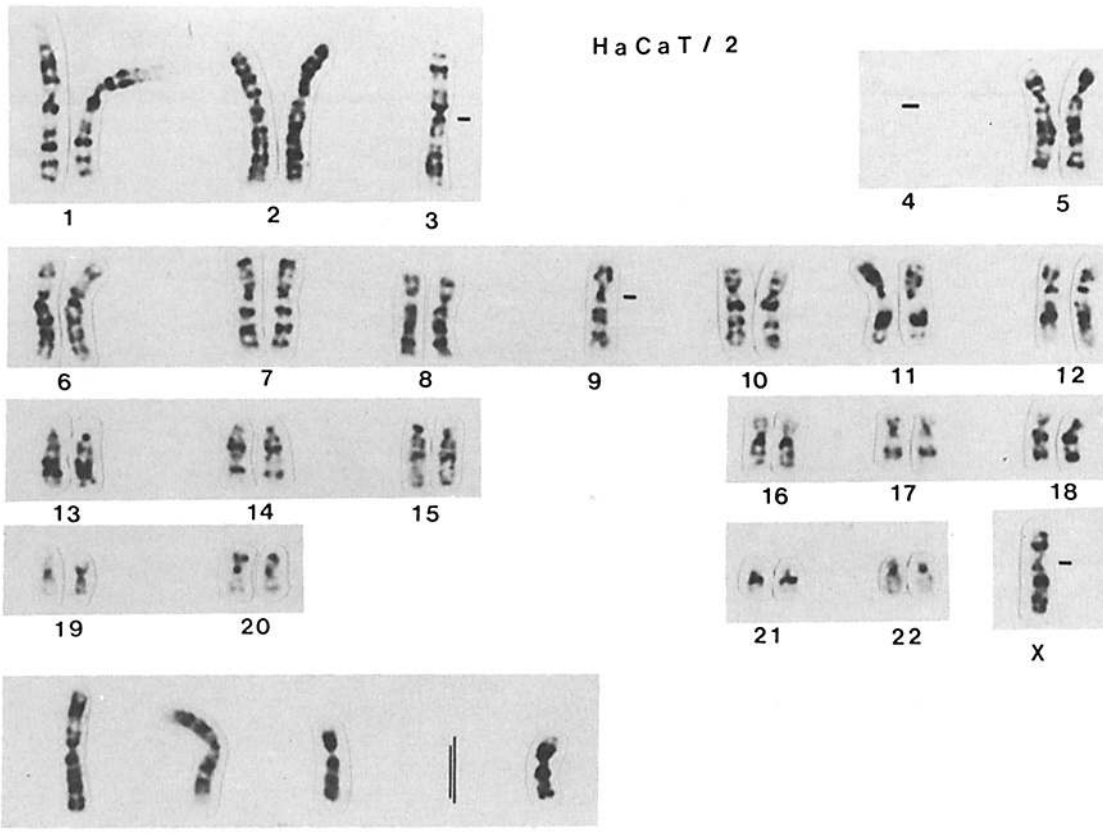
\* M1 t(3,4)(qter → q11:q11 → qter), M2 i(9)(qter → q11:q11 → qter), M3 del(4)(q28 → qter), M4 (4,18)(pter → p11:q11 → qter).

‡ Metaphases contained only either M3 or M4 alternatively.

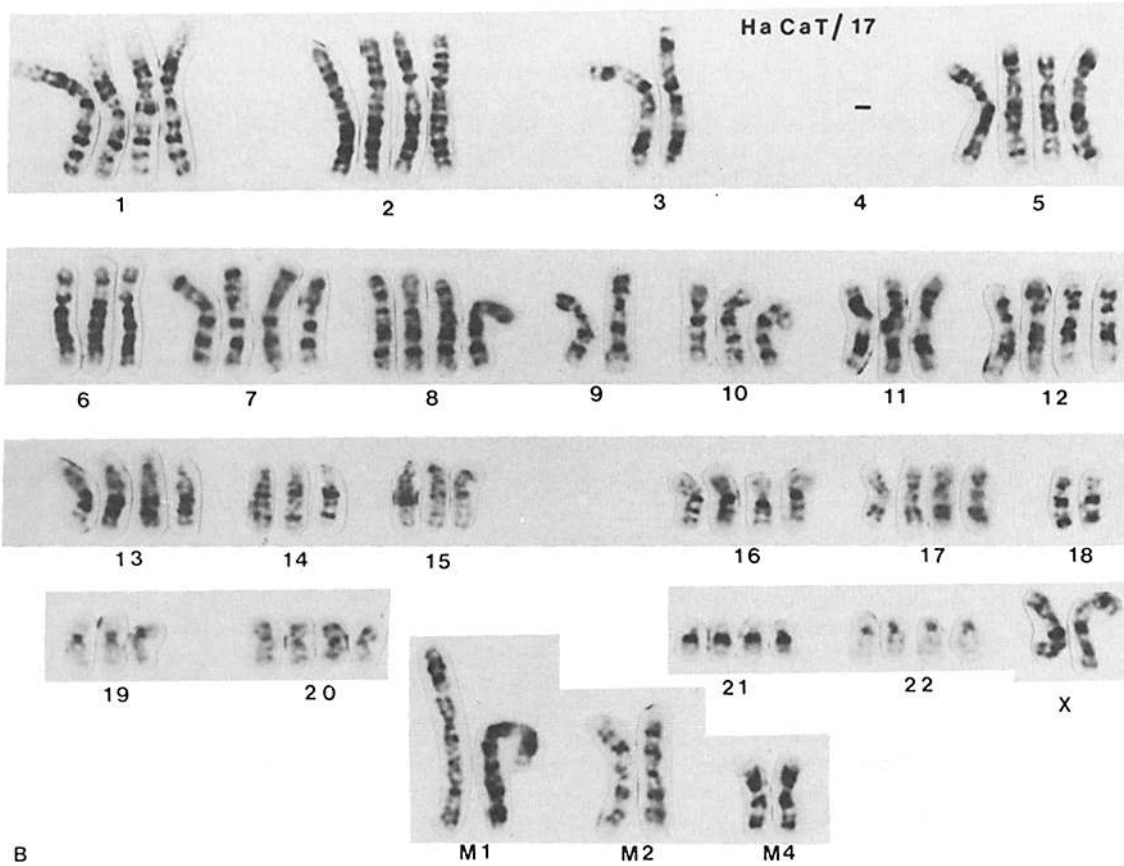
§ M5: dup(1)(q 23.1 → 25.3); M6: t(15,22); M7: dup(6)(p22); M8: dup(17)(q23.1 → q25.3).

¶ Present in 47, 30, 30, and 15% and

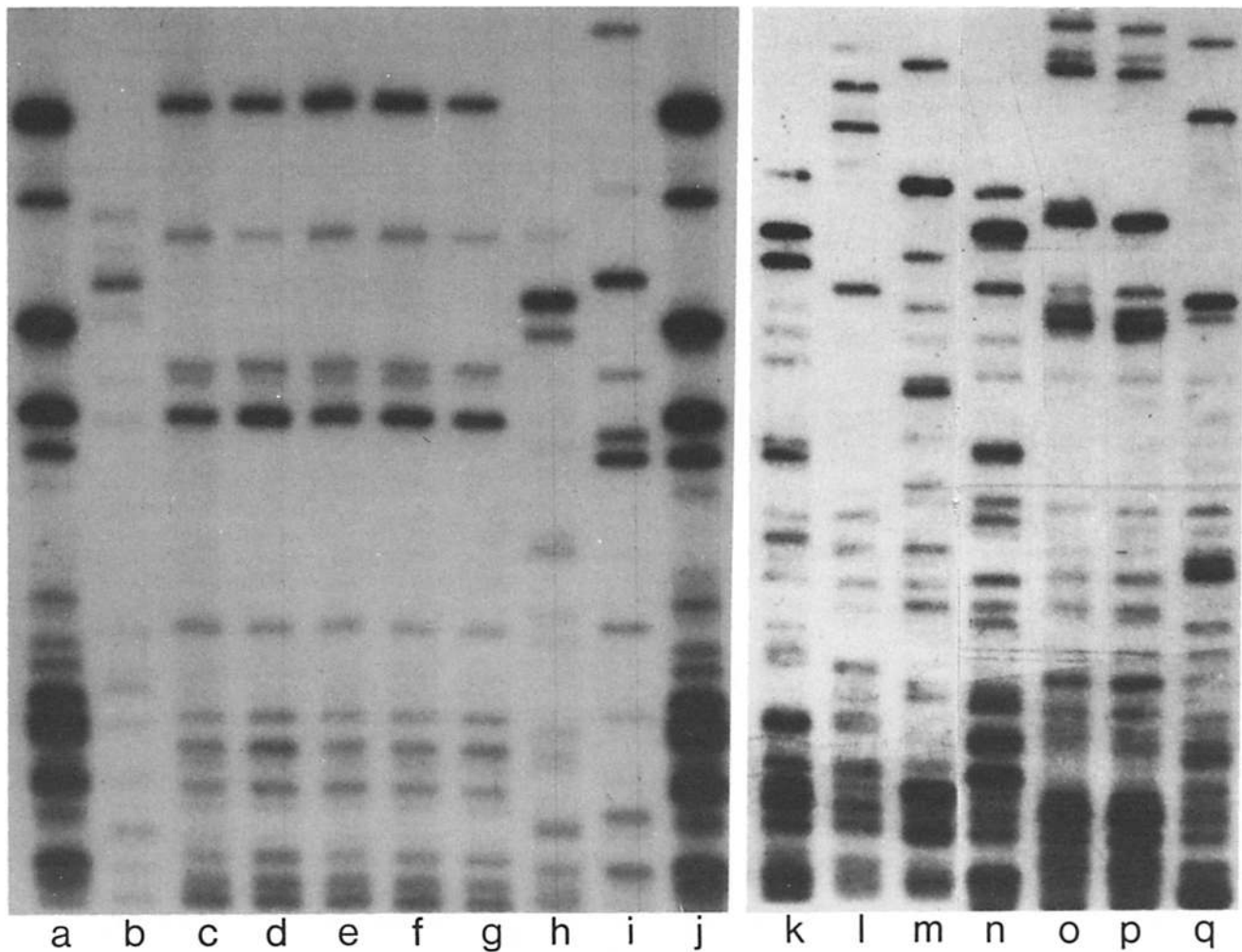
† Present in 50, 30, 25, and 25% of the metaphases, respectively.



A M1 M2 M3 M4 (P.5)



B



**Figure 3.** Southern blot analysis of DNA from tissue and cell samples hybridized with the hypervariable minisatellite probe 33.15. (lane *a*) and standard DNA from an unrelated subject (lane *j*); (lane *b*) lymphocyte DNA from an unrelated patient; (lane *c*) skin biopsy of donor patient (EB); (lanes *d-g*) HaCaT cells from passages 45, 6, 16, and 79, respectively; (lane *h*) HaSV cells, human keratinocyte line immortalized by transfection with SV40 DNA, passage 18; (lane *i*) HeLa cells; (lane *k*) HaSV cells, passage 43; (lane *l*) SCL-I cells and (lane *m*) SCL-II cells, established from squamous cell carcinomas of the skin; (lane *n*) TR 126 cells; (lane *o*) TR 131 cells; (lane *p*) TR 138 cells; (lane *q*) TR 146 cells (lanes *n-q* established from squamous cell carcinomas of the head and neck; see Boukamp et al., 1985).

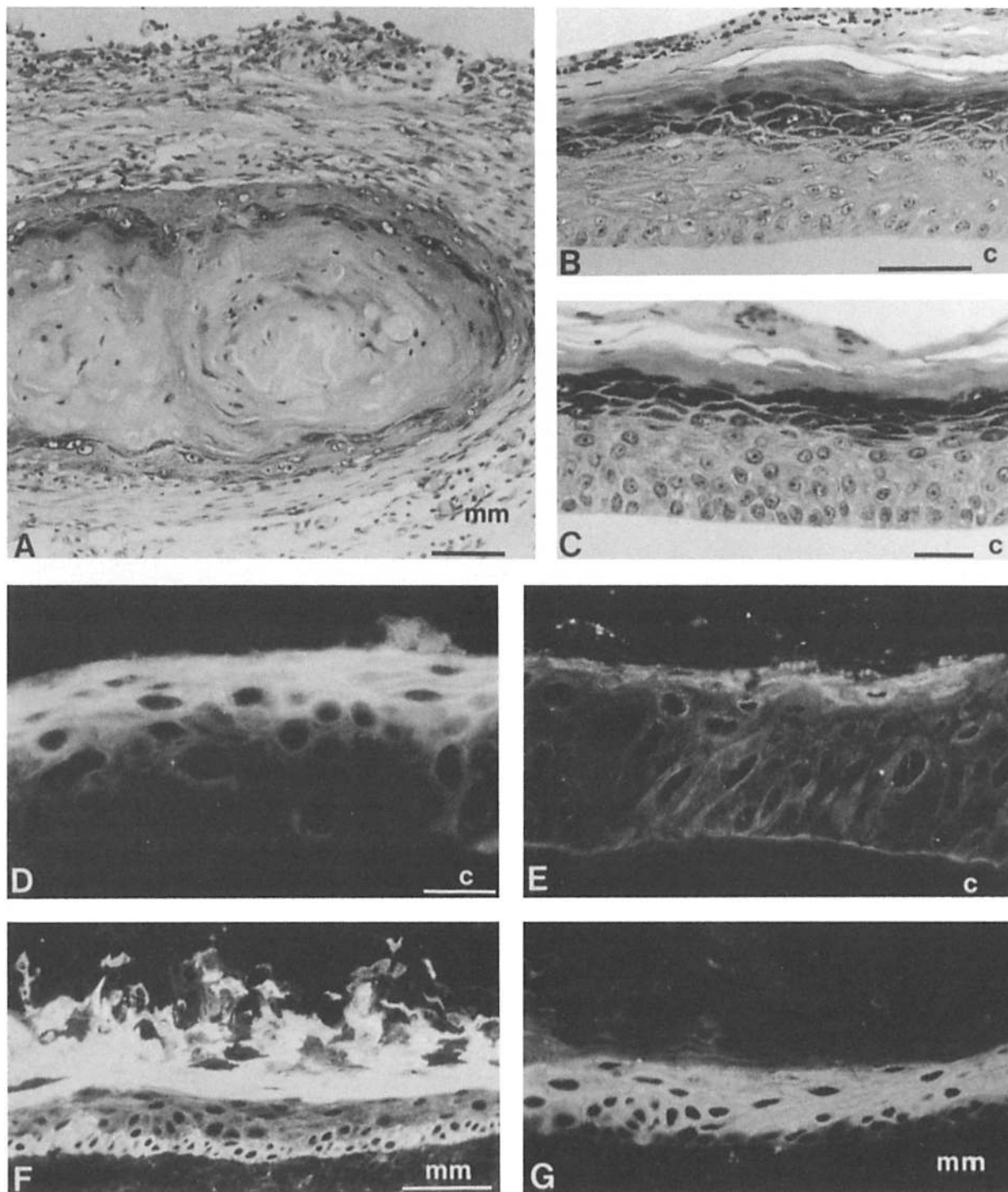
tions from regular tissue architecture and an increasing transition from an ortho- to a parakeratotic stratum corneum (containing nuclear remnants). However, a distinct stratum granulosum (usually restricted to orthokeratinization) was present in all transplants.

Using other differentiation markers in an effort to further substantiate the histological findings, transplants of HaCaT cells at different passage levels were analyzed both biochemically and by immunofluorescence. Involucrin, a major precursor protein of the cross-linked envelopes in the stratum corneum, was clearly restricted to the upper flattened layers of the transplants (Fig. 4 *D*), a characteristic feature of long-term transplants of normal adult keratinocytes (Watt et al., 1987). Also filaggrin, the major component of kerato-

hyalin granules, was detected in the two uppermost living layers (Fig. 4 *E*), i.e., the stratum granulosum. Moreover, basal and suprabasal cell compartments were clearly distinguishable (as in the epidermis) by a monoclonal antibody (Pab421) specific for epidermal basal cells (Fig. 4 *F*) and by a polyclonal antibody specific for one of the epidermal suprabasal keratins (57 kD, No. 10/11 according to Moll et al., 1982; Fig. 4 *G*).

These findings correlated well with keratin analysis by one- and two-dimensional gel electrophoresis (NEpHGE/SDS-PAGE). When transplants of HaCaT cells (Fig. 5 *A*) were compared with those of normal cells derived from adult foreskin (Fig. 5 *B*) very similar patterns were observed. As in the human epidermis (adult thigh skin, Fig. 5 *C*) the

**Figure 2.** G-banded karyotype of the HaCaT cells. (*A*) At passage 2 the cells revealed a hypodiploid stem line (mode of 44 chromosomes) with three individual marker chromosomes: M1, t(3q4q); M2, i(9q); M3, del(4q28) (in 100% of the metaphases) and monosomy X. At passage 5 the marker M4 t(4p18q) appeared. (*B*) At passage 17 the stem line shifted to hypotetraploidy (mode of 82). The markers M1, M2, and M4 were present in two copies while M3 had disappeared.



**Figure 4.** Histological sections of 2-wk-old HaCaT transplants. (A) Suspensions of HaCaT cells, transplanted directly onto the muscle fascia of nude mice, develop cysts within the mouse mesenchyme (*mm*) with orderly stratified keratinizing epithelium. (B) HaCaT cells transplanted as intact cultures on collagen substratum (*c*) form a regular epidermis comparable (C) to normal skin keratinocytes. Immunofluorescence of HaCaT transplants on collagen (D and E) and directly on the muscle fascia (F and G). (D) Antiserum against human involucrin shows a membrane-bound reaction in the upper flattened living cell layers of the epithelium. (E) Filaggrin staining (spotlike fluorescence) apparent in the uppermost living cell layers corresponding to the keratohyalin granules of the stratum granulosum. (F) The lowermost cell layer of the epithelium lining the cysts in the mouse mesenchyme (*mm*) is stained with a basal cell-specific monoclonal antibody (Pab421). The stratum corneum is unspecifically labeled by FITC. (G) The suprabasal cell layers of the epithelium are stained with sequence-specific polyclonal antibodies against the high molecular mass acidic 57-kD (No. 10) keratin. Bars: (A) 100  $\mu$ m; (B, F, and G) 50  $\mu$ m; (C, D, and E) 25  $\mu$ m.



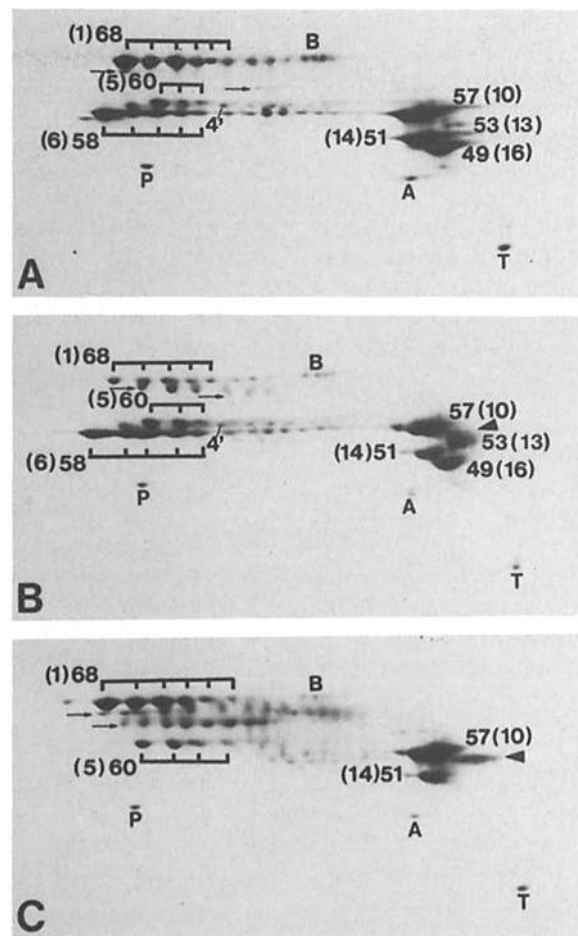
suprabasal basic 68-kD and acidic 57-kD keratins (Nos. 1 and 10/11), as well as the basal basic 60-kD and acidic 51-kD keratins (Nos. 5 and 14, respectively) were strongly expressed. Furthermore, both HaCaT and normal cell transplants maintained the hyperproliferative set of basic 58-kD and acidic 49-kD keratins (Nos. 6 and 16, respectively). Minor components were keratin No. 4 (presumably) and No. 13. Keratin Nos. 6 and 16 are regularly expressed in culture (together with the basal keratin pair and No. 17), but in epidermis only in the hyperplastic state (Sun et al., 1983). These observations are compatible with the general (hyperplastic) morphology of transplants and consistent with previous data on transplanted mouse keratinocytes (Breitkreutz et al., 1984, 1986).

Keratin No. 13, abundant in foreskin and derived cultures but absent in adult epidermis, was prominent in HaCaT cultures (data not shown). Also present, although mostly in traces, were keratin Nos. 7, 8, 15, 18, and 19. These simple epithelial-type keratins were highly inducible by vitamin A in vitro but absent in transplants. Interestingly, in vitamin A-deprived cultures (delipidized serum) HaCaT cells expressed keratin patterns comparable to those of transplants (our unpublished data). This further stresses the similarity of the differentiation behavior of HaCaT cells to that of normal keratinocytes. Generally this keratinization pattern is in marked contrast to SV40-transformed keratinocyte lines where small or simple epithelial-type keratins such as 7, 8, and 18 or 19 clearly predominate (Bernard et al., 1985; Steinberg and Defendi, 1983; Banks-Schlegel and Hawley, 1983; our unpublished observations) and where the suprabasal keratin pair is apparently not induced in vivo.

## Discussion

Herein we have reported the spontaneous transformation in vitro of human keratinocytes from histologically normal adult body skin. The apparently immortalized but highly differentiated cell line was named HaCaT to indicate the origin and initial growth conditions (see Experimental Procedures). The line is clonal in origin as indicated by specific and stable cytogenetic markers, has a transformed phenotype in vitro but is not tumorigenic, and is noninvasive in vivo. This clearly demonstrates, in accord with earlier studies (Azzarone et al., 1976; Danes and Surano, 1982; Mukherji et al., 1984; Thielmann et al., 1983; Revoltella et al., 1986; Baden et al., 1987a, b; Nagasawa et al., 1987), that spontaneous transformation of human cells in vitro can occur, although the number of reported cases is still rather low.

The relatively low success rate for transformation of human cells in vitro has mainly been attributed to the higher genetic stability of human cells as compared with rodent cells (DiPaolo, 1983; Sager et al., 1983). Although detailed studies on chromosomal alterations using banding techniques are rare, it has been shown that cytogenetically abnormal clones in cultures of adult human skin fibroblasts are by no means uncommon (Littlefield and Mailhes, 1975; Harden et al., 1976; Nagasawa et al., 1987). Our preliminary experiments with normal human keratinocytes indicate that numerical (mainly polyploidization) and nonspecific structural chromosomal changes are present in early and late primary cultures as well as subcultures (R. T. Petrusevska, unpublished observations). Other studies with cultured rat embryo



**Figure 5.** Keratins expressed in cell transplants (*A* and *B*) and human epidermis (*C*) separated on two-dimensional gels. In both transplants (*A*) of HaCaT cells and (*B*) those of normal human keratinocytes the suprabasal (68 kD, No. 1 and 57 kD, No. 10/11) and basal keratins (60 kD, No. 5 and 51 kD, No. 14) are present at about equal levels. In addition, the hyperproliferative set (58 kD, No. 6 and 49 kD, No. 16) is expressed, which is generally lacking in normal adult (thigh) epidermis (*C*). The varying degrees of turnover products (derived from keratin No. 1, marked by small arrows; also from No. 10/11, indicated by some slight trailing to the right, arrowhead) between the transplants are also observed in normal epidermis of different individuals and/or depending on body location. The minor spots in the neutral range (*A*, possibly also *B*; marked 4') might represent some keratin No. 4 (assessed by comigration with authentic sample). Separation in the first dimension was achieved by NEpHGE (right to left) and in the second dimension by SDS-PAGE (7.5–17.5% acrylamide gradient). Markers included are: *B*, BSA (68 kD, pI 6.4); *P*, 3-phosphoglycerate kinase (43 kD, pI 7.4); *A*,  $\alpha$ -actin (42 kD, pI 5.4); *T*, tropomyosin (36 and 38 kD, pI 4.9).

and monkey fibroblasts indicated that elevated temperature (as used here to establish the cell line) accelerates the occurrence of (spontaneous) chromosomal aberrations (Marczynska et al., 1980). We are currently investigating this effect in primary and low passage human keratinocyte cultures.

The significance of chromosomal alterations in different stages in cell transformation is not yet fully established. The early appearance and the stability of the distinct cytogenetic

changes (particularly the unbalanced hypodiploidy) in the HaCaT cells strongly indicate that they are essential for the early disturbances in growth control resulting in prolonged or unlimited life span. The subsequent occurrence of a hypotetraploid HaCaT subclone and its correlation with improved growth capacity in vitro has also been previously observed in two human skin carcinoma cell lines (Tilgen et al., 1983) and in transformed mouse keratinocyte lines (Fusenig et al., 1985). Therefore, polyploidization, which further contributes to genomic imbalance and altered gene expression, may be crucial for the acquisition of autonomous growth in culture but is certainly not sufficient for malignant conversion. Very recently, another spontaneous human keratinocyte cell line (from foreskin) that exhibited as a sole karyotypical abnormality a trisomy of chromosome No. 8 has been described (Baden et al., 1987a, b). The fact that this near diploid cell line still depends on 3T3 feeder support for continuous growth, further supports the hypothesis that polyploidization is crucial for autonomous growth in vitro.

Because spontaneous immortalization is still generally considered to not occur in human cells, it was critical to prove the identity of the HaCaT cells with the original donor and to exclude cross-contamination with epithelial human cell lines used in our laboratory. Although the cytogenetic data documenting individual marker chromosomes had virtually excluded possible cross-contamination, additional proof was highly desirable. DNA fingerprinting on Southern blots using hypervariable minisatellite probes (Jeffreys et al., 1985) seemed to be the method of choice, although it had not previously been demonstrated whether chromosomal alterations would influence the applicability of this technique. The results unequivocally proved that despite the transformed phenotype and multiple structural chromosomal changes the DNA fingerprint pattern remained essentially identical at various passage levels and was clearly identical to a DNA sample from the patient. Moreover, the nonidentity of a variety of different cell lines tested as well as DNA fingerprints from 200 unrelated individuals (data not shown) clearly demonstrated the unique DNA fingerprint pattern of HaCaT cells and their original donor.

These findings underline the power of the DNA fingerprinting technique for unequivocal identification of cultured cells and the verification of their original donor, irrespective of the tissue used as DNA source (e.g., lymphocytes, skin tissue sample, and keratinocytes). Thus, these results provide new standards for the identification of human cells in vitro irrespective of time in culture and genetic alterations.

It is a widely accepted postulate that alterations in differentiation are essential for the transformation process and that malignancy and differentiation are inversely correlated (Broders, 1932). Cell lines derived from skin carcinomas usually exhibit defects in their differentiation capacity, although with considerable variations (Rheinwald and Beckett, 1980; Boukamp et al., 1985). In accord with this, immortalization of human keratinocytes with SV40 was accompanied by drastic alterations in differentiation potential (Banks-Schlegel and Howley, 1983; Steinberg and Defendi, 1983; Bernard et al., 1985). It has therefore been stated that "inhibition of keratinization might be an early step in carcinogenesis." Steinberg and Defendi (1983) and concluded that the loss in differentiation capacity in cell lines would be "the price one has to pay for immortalization." However, these

properties of the SV40-transformed cells might be virus-related rather than linked to transformation per se. This is in line with our own data on SV40-immortalized human skin keratinocytes (our unpublished results) and other studies on virus-transformed keratinocytes (Yuspa et al., 1983; Weissman and Aaronson, 1985).

In contrast to these virally transformed human cells, the HaCaT cells, although immortal, have largely retained their capacity to reconstitute a well structured epidermis after transplantation in vivo. The virtually normal degree of morphologic differentiation was further substantiated by the regular spatial distribution of epidermal differentiation products. In addition, the pattern of keratin expression, including the suprabasal epidermal keratins (Nos. 1 and 10), was almost identical to those seen in transplants of normal keratinocytes. We have previously reported similar findings on a series of spontaneously transformed mouse keratinocyte lines (Breitkreutz et al., 1986). The HaCaT cells also maintained these properties at higher passage levels, even though differentiation in vitro (stratification and squame formation) gradually decreased and slight alterations in tissue architecture occurred in vivo. In addition, HaCaT cells from passage 80 or higher showed a comparable induction of suprabasal keratins in response to vitamin A depletion in the culture medium (our unpublished results). In this way, the HaCaT cell line is closely approximated to normal keratinocytes, and thus offers a suitable model to study regulatory mechanisms in the process of differentiation of human epidermal cells. Moreover, since HaCaT cells could be reproducibly and efficiently transfected with the human Ha-ras-1 oncogene (EJ), giving rise to clones with abnormal growth properties in vivo, including tumorigenesis (Fusenig et al., 1987; Boukamp, P., D. Breitkreutz, E. Stanbridge, P. Cerutti, and N. Fusenig, manuscript in preparation), this cell line provides a valuable model system for the study of the role of oncogenes and other factors in the process of malignant conversion of human epithelial cells.

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