Normal Growth and Differentiation in a Spontaneously Immortalized Near-Diploid Human Keratinocyte Cell Line, NIKS

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We report the isolation and characterization of a spontaneously immortalized human keratinocyte cell line, NIKS. The cell line is not tumorigenic in athymic nude mice and maintains cell-type-specific requirements for growth *in vitro*. NIKS cells express steady-state levels of transforming growth factor-α, transforming growth factor-β1, epidermal growth factor receptor, c-*myc*, and keratin 14 mRNAs comparable with the parental BC-1-Ep keratinocyte strain. BC-1-Ep and NIKS keratinocytes produce similar levels of cornified envelopes and nucleosomal fragmentation in response to loss of substrata attachment. DNA fingerprinting results confirm that the NIKS cells originated from the parental BC-1-Ep keratinocytes. NIKS cells contain 47 chromosomes due to an extra isochromosome of the long arm of chromosome 8, and the near-diploid karyotype appears to be stable with repeated passage. A fully stratified squamous epithelium is formed by the NIKS keratinocytes in organotypic culture. Ultrastructural analysis of both the parental and immortalized keratinocytes reveals abundant desmosomes, hemidesmosomes, and the production of a basal lamina. Our findings with the NIKS cells support the observation that spontaneous immortalization is not linked to alterations in squamous differentiation or the ability to undergo apoptosis. The NIKS human keratinocyte cell line is an important new tool for the study of growth and differentiation in stratified squamous epithelia. *Key words: hemidesmosomes/nucleosomal fragmentation/organotypic culture/trisomy. J Invest Dermatol 114:444–455, 2000*

Cultivated cells isolated from disaggregated human skin have been used for the study of growth and differentiation of keratinocytes for over two decades (reviewed in Leigh *et al*, 1994). Human foreskin keratinocytes cultured in the presence of a 3T3 mouse embryo fibroblast feeder layer or in serum-free medium formulations exhibit sustained growth for approximately 80 population doublings prior to senescence (reviewed in Leigh and Watt, 1994). Human keratinocytes cultured under these conditions are capable of expressing differentiation-specific proteins, such as involucrin and keratins K1 and K10, in a position-specific manner (reviewed in Fuchs and Weber, 1994). Although features of squamous differentiation and limited stratification are consistently observed in monolayer cultures of keratinocytes, normal tissue architecture is not evident. Full stratification and histologic differentiation can be achieved by the use of three-dimensional organotypic culture methods where keratinocytes are grown on the surface of collagen gels containing dermal fibroblasts (Bell *et al*, 1979; and reviewed in Parenteau *et al*, 1992; Fusenig, 1994).

Unlike murine keratinocytes, spontaneous immortalization of human keratinocytes is rarely observed. Three spontaneously immortalized human keratinocyte lines have been reported to date (Baden *et al*, 1987; Boukamp *et al*, 1988; Rice *et al*, 1993). Two of the lines, NM 1 and SIK, arose from keratinocytes isolated from neonatal foreskins (Baden *et al*, 1987; Rice *et al*, 1993). The third, HaCaT, was isolated from adult epidermis at the periphery of a malignant melanoma (Boukamp *et al*, 1988). Long-lived keratinocyte lines, such as the HKC-N2 cell line and others, have been derived from commercially supplied epidermal keratinocytes isolated from adult mammary tissue; however, these lines have not been demonstrated to be immortal (Weaver *et al*, 1991). The HaCaT cell line is the most extensively characterized of the three spontaneously immortalized human keratinocyte cell lines. Although the HaCaT cells exhibit the transformed phenotype of anchorage-independent growth in soft agar, they are not tumorigenic in immunocompromised mice. When cultivated in organotypic culture and transplanted onto immunocompromised mice, HaCaT keratinocytes generate a stratified epithelia, albeit at a slower rate than normal human keratinocytes, which exhibits features of squamous differentiation (Breitkreutz *et al*, 1998; Boelsma *et al*, 1999; Schoop *et al*, 1999). The cytogenetic characteristics of the three spontaneously immortalized human keratinocyte lines vary considerably. The HaCaT cell line exhibits early and distinct numerical as well as structural karyotypic alterations whereas karyotypic analysis of the NM 1 cell line, derived from a pool of foreskins, showed only trisomy of the long arm of chromosome 8.

We have isolated and characterized a spontaneously immortalized human keratinocyte cell line, NIKS (*N*ormal *I*mmortal *K*eratinocyte*S*), arising from the BC-1-Ep strain of normal human neonatal foreskin keratinocytes. The NIKS keratinocyte line

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maintains cell-type-specific requirements for growth *in vitro* and expresses steady-state levels of transforming growth factor (TGF)-α, transforming growth factor-β1, epidermal growth factor receptor, c-*myc*, and keratin 14 mRNAs comparable with the parental BC-1-Ep keratinocyte strain from which it arose. The cell line is nontumorigenic and produces a fully stratified squamous epithelium in organotypic culture. Our findings provide additional evidence suggesting that spontaneous immortalization is not tightly linked to alterations in squamous differentiation or the ability to undergo apoptosis. The NIKS human keratinocyte cell line represents an important new tool for the study of growth and differentiation in stratified squamous epithelia.

MATERIALS AND METHODS

Cell culture Normal keratinocytes (BC-1-Ep) were isolated from newborn human foreskin. Keratinocyte cultures were established by plating aliquots of a single cell suspension in the presence of mitomycin C-treated Swiss mouse 3T3 fibroblasts as previously described (Allen-Hoffmann and Rheinwald, 1984). The standard keratinocyte culture medium was composed of a mixture of Ham's F-12 medium/Dulbecco's modified Eagle's medium (DME) (3:1, final calcium concentration 0.66 mM), supplemented with 2.5% fetal bovine serum (FBS), 0.4 μg hydrocortisone (HC) per ml, 8.4 ng cholera toxin (CT) per ml, 5 μg insulin (Ins) per ml, 24 μg adenine per ml (Ade), 10 ng epidermal growth factor (EGF) per ml, 100 units penicillin, and 100 μg streptomycin (P/S) per ml. The cells were subcultured at weekly intervals at 3×10^5 cells per 100 mm tissue culture dish (approximately a 1:25 split) with a mitomycin C-treated feeder layer. NIKS appeared at passage 16. NIKS cells tested negative for mycoplasma at passage 55 (Wisconsin State Laboratory of Hygiene, Madison, WI). Recombinant human EGF and TGF-β1 was obtained from R&D Systems (Minneapolis, MN).

Chromosomal analysis Cells in log phase growth were arrested in metaphase with 50 ng Colcemid per ml, then trypsinized and collected by centrifugation. After removal of the media and trypsin, the cells were suspended in a hypotonic 75 mM KCl solution for 20 min, fixed with 3:1 methanol/acetic acid three times and dropped on to glass slides. Slides were aged for 2 wk and G-banded by immersing in 0.5% trypsin for 25–30 s followed by staining with Giemsa. In each sample, the chromosomal identities and aberrations were determined in well-spread G-banded metaphases by photographic analysis. At least two to three keratinocytes from 20 to 50 metaphases were prepared and examined at passages 3, 31, and 54. A band-by-band analysis of each chromosome was done in at least 10 cells for each passage, so that any rearrangements would be detected. In addition, fluorescent *in situ* hybridization (FISH) using centromere probes for chromosomes 1 and 8 (Oncor, Gaithersburg, MD) were used to study passage 3 and passage 18 cells. Five hundred passage 3 and 100 passage 18 cells were examined by FISH analysis.

DNA fingerprinting DNA was isolated from keratinocytes using Qiagen QIAamp Blood Kit (Qiagen, Santa Clarita, CA). DNA fingerprint analysis used the GenePrint Fluorescent STR System according to protocols recommended by the manufacturer (Promega, Madison, WI). The 12 primer pairs are divided into three quadriplexes (CTTv, FFFL, and GammaSTR). Each quadriplex was amplified in separate PCR reactions using 25 ng of DNA as template. PCR products were electrophoresed on 42 cm × 33 cm × 0.4 mm polyacrylamide gels in a BRL sequencing apparatus (Life Technologies, Gaithersburg, MD). Gels were then scanned on a Hitachi FMBIO II Fluorescent Scanner at 505 nm at a resolution of 300 dpi × 300 dpi with a sampling repeat of 800.

Growth of NIKS cells in athymic mice NIKS cells were injected into athymic nude mice to assess tumorigenicity. A suspension of 5×10^6 cells in 100 μl Ham's F-12 was injected subcutaneously into the flanks of six athymic nude mice. As a negative control, the parental BC-1-Ep (passage 6) cells were injected at 3×10^6 cells per 100 μl Ham's F-12 medium. As a positive control, tumorigenic SCC4 cells were injected at 3×10^6 cells per 100 μl Ham's F-12. Mice were weighed and tumors measured 26 d later.

Anchorage independent growth assays Anchorage-independent growth was determined by a methylcellulose/agar assay (Allen-Hoffmann *et al*, 1990) and an agar assay. For the methylcellulose/agar assay, NIKS cells (passages 34, 37, and 39) were suspended at 6×10^3 cells per ml in 1.68% methylcellulose (4000 centipoises; Fisher Scientific, Fairlawn, NJ) in Ham's F-12/DME (3:1, final calcium concentration 0.66 mM) supplemented with 2.5% FBS, 0.4 μg HC per ml, 8.4 ng CT per ml, 5 μg Ins per ml, 24 μg Ade per ml, 10 ng EGF per ml, 100 units penicillin, and 100 μg streptomycin per ml. For the agar assay, NIKS cells were suspended at 6×10^3 cells per ml in 0.3% Noble agar in Ham's F-12/DME (3:1, final calcium concentration 0.66 mM) containing the previously described supplements. A 1 ml aliquot of each of these mixtures containing NIKS cells was pipetted on to 1 ml base layers of 0.5% Noble agar in a 9 cm^2 gridded tissue culture dish. NRK cells, clone 49F (American Type Culture Collection, CRL 1570) were used as a positive control for both the methylcellulose/agar and agar assays as previously described (Sachsenmeier *et al*, 1996). Plates were then incubated at 37°C in a humidified 5% CO_2 atmosphere. NRK colonies ⩾ 60 μm in diameter were counted unfixed and unstained at 1 wk and 3 wk after plating for the methylcellulose and agar assays, respectively. NIKS keratinocytes were stained 8 wk after plating for both assays.

Suspension in semi-solid media For suspension studies, preconfluent cultures were removed from culture dishes with 0.5 mM ethylenediamine tetraacetic acid (EDTA), 0.1% trypsin and washed in serum-containing medium to inactivate any residual trypsin. After a short centrifugation ($440 \times g$ for 5 min), cells were resuspended at 1×10^6 cells per ml in serum-free and additive-free Ham's F-12/DME (3:1, final calcium concentration 0.66 mM) supplemented with P/S and made semi-solid with 1.68% methylcellulose (4000 centipoises; Fisher Scientific) as described previously (Sadek and Allen-Hoffmann, 1994). Cells were recovered from suspension by repeated dilution with serum-free medium and centrifugation ($400 \times g$ for 10 min). Following one rinse with phosphate-buffered saline (0.137 m NaCl; 2.7 mM KCl; 8.1 mM Na_2HPO_4; 1.4 mM KH_2PO_4; pH 7.2 (PBS), cells were either resuspended in PBS (pH 7.2) to assay for cornified envelope formation or lysed in SLS buffer [50 mM Tris; 10 mM EDTA, pH 8.0; 0.5% (wt/vol) sodium lauroyl sarcosine] to determine DNA fragmentation. Controls consisted of adherent keratinocytes treated for similar times in Ham's F-12/DME (3:1, final calcium concentration 0.66 mM) supplemented with P/S.

Northern analysis Cells were grown in standard keratinocyte culture medium with a mitomycin C-treated 3T3 feeder layer. The feeder layer was removed 24 h prior to RNA isolation with 0.02% EDTA in PBS. Poly(A)$^+$ RNA was isolated from logarithmically growing cells as previously described (Sadek and Allen-Hoffmann, 1994). Poly(A)$^+$ RNA was electrophoresed in a 1.2% agarose gel containing formaldehyde and electroblotted to a Zeta-probe membrane (BioRad Laboratories, Richmond, CA). The membrane was prehybridized and the hybridized in the presence of a random primer [^{32}P]-dCTP-labeled cDNA probe as recommended by the supplier. The cDNA probes used for detection include glyceraldehyde-3-phosphate dehydrogenase, pGPDN5 (Fort *et al*, 1985), TGF-β1 (Sharples *et al*, 1987), EGF receptor (Xu *et al*, 1984), keratin 14 (kind gift from Dennis Roop), TGF-α (Kudlow *et al*, 1989), and c-*myc* (Miyamoto *et al*, 1985).

Cornified envelope (CE) formation Keratinocytes were removed from culture plates and recovered from suspension as described above. Cells from each treatment were counted and resuspended in triplicate at 10^6 cells per ml in PBS (pH 7.2) containing 1% sodium dodecyl sulfate and 20 mM dithiothreitol. Samples were boiled for 5 min in a water bath and cooled to room temperature. DNase (0.5 mg per ml) was added and CE counted using a hemacytometer. CE formation was calculated as a percentage of input cells.

Analysis of nucleosomal DNA fragmentation DNA was isolated and labeled as previously described (Sachsenmeier *et al*, 1996). Briefly, 2.5×10^6 cells were lysed in 500 μl of 50 mM Tris, 10 mM EDTA (pH 8.0), and 0.5% (wt/vol) sodium lauroyl sarcosine. The lysate was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol) and ethanol precipitated. The DNA was dissolved in 20 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and quantitated by absorption at 260 nm. Intact and fragmented DNA was 3′ end-labeled with [α^{32}P]-ddATP using terminal dideoxynucleotidyl exotransferase as previously described (Tilly and Hsueh, 1993). One-half of each labeled sample was loaded on to a 1.5% agarose gel and electrophoresed. Gels were dried with heat using an SE 1200 Easy Breeze (Hoefer Scientific, San Francisco, CA) and exposed to Kodak BioMax MR film.

Isolation of p53 transcripts p53 cDNAs were generated using reverse transcription–PCR on total RNA isolated from the BC-1-Ep and NIKS cells. Cultured cells were harvested from a single 100 mm dish using a plastic scraper and total RNA was isolated using TRIzol reagent according to protocols recommended by the manufacturer (Life Technologies).

"Ready To Go You-Prime-First-Strand Beads" were used for reverse transcription reactions (Amersham Pharmacia Biotech, Piscataway, NJ). Reactions contained approximately 4 μg of total RNA and 40 pmol of the p53 reverse transcription primer (5′-GCTTCTGACGCACACC-TATTG-3′). The entire reverse transcription reaction was used in subsequent PCR to amplify the full-length coding region using a p53 forward primer: (5′-AGAGATGGGGGAGGGAGGCTGTCA-3′) and a reverse primer: (5′-GCTCCGGGGACACTTTGCGTTCG-3′).

Sequence analysis of p53 The sequence of the coding region of p53 was determined by direct sequencing of reverse transcription–PCR products. Products from the reverse transcription–PCR reactions were run on 1% agarose gels and purified using Qiagen QIAquick Gel Extraction Columns (Qiagen, Valencia, CA). The sequence was then determined using the above-mentioned p53 forward and reverse primers and the Perkin-Elmer ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Norwalk, CT). Sequence analysis was performed using the DNASTAR software package (DNAStar, Madison, WI).

Formation of organotypic cultures Organotypic cultures were grown as previously described (Parenteau, 1994) in specialized culture chambers (Organogenesis, Canton, MA) with the following changes: a collagen base was formed by mixing normal human neonatal fibroblasts, strain CI-1-F, with type I collagen in Ham's F-12 medium containing 10% FBS and P/S. The collagen base was allowed to contract for 5 d. The parental keratinocytes, BC-1-Ep (passage 5), and the NIKS keratinocytes (passage 38) were plated on the contracted collagen base at 3.5×10^5 cells in 50 μl of a mixture of Ham's F-12/DME (3:1, final calcium concentration 1.88 mM) supplemented with 0.2% FBS, 0.4 μg HC per ml, 8.4 ng CT per ml, 5 μg Ins per ml, 24 μg Ade per ml, and P/S. Cells were allowed to attach 2 h before flooding culture chambers with media (day 0). On days 1 and 2 cells were refed. On day 4, cells were lifted to the air interface with cotton pads and switched to cornification medium containing Ham's F-12/DME (3:1, final calcium concentration 1.88 mM) supplemented with 2% FBS, 0.4 μg HC per ml, 8.4 ng CT per ml, 5 μg Ins per ml, 24 μg Ade per ml, and P/S. Cells were fed cornification medium every 3 d. On day 15, organotypic cultures were fixed with fresh Karnovsky's fixative consisting of 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 3 h. Before removing the culture media, fixative was gently added to the cells on top of the organotypic cultures to prevent cornified layers from dislodging. The culture media were then aspirated and the culture wells filled with fixative. Organotypic cultures were cut in half with one-half processed for light microscopy and the other half for electron microscopy.

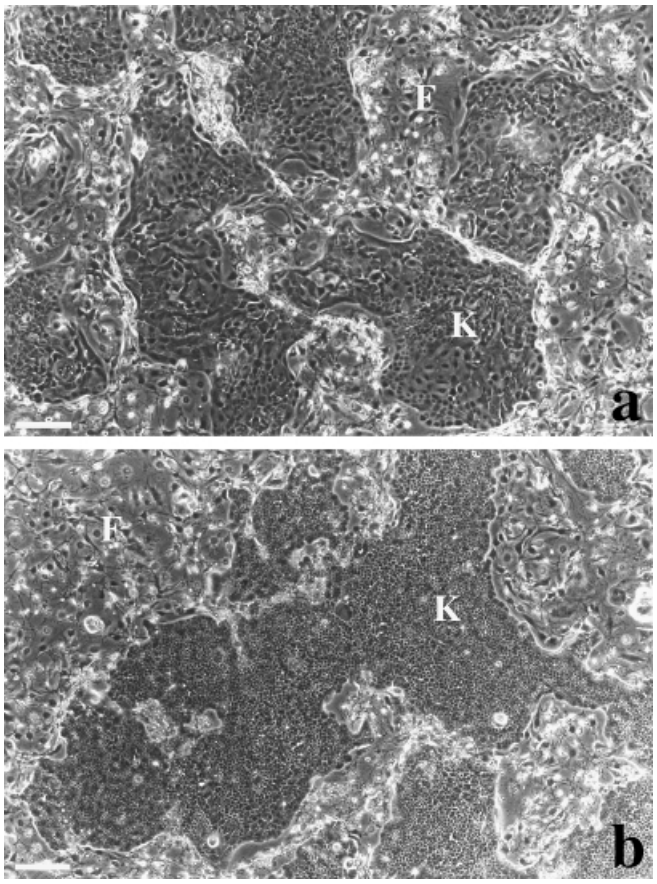

Figure 1. Culture of NIKS cells. BC-1-Ep cells were grown and serially subcultured in standard keratinocyte growth media. BC-1-Ep cells at passage 15 (*a*) appeared large and flat indicating senescence. At passage 16, however, a new population of cells with a small, packed morphology appeared. At passage 17 (*b*) these cells completely replaced the previous population. The cells were serially subcultured until passage 58 (372 d). *Scale bar*: 300 μm.

Tissue sectioning Fixed organotypic cultures were embedded in paraffin, sectioned and stained with hematoxylin and eosin by Surgical Pathology, University Hospital (Madison, WI). Stained sections were viewed and photographed using an Olympus IX-70 microscope equipped with a 35 mm camera.

Electron microscopy Fixed organotypic cultures were washed three times with 0.1 M cacodylate buffer, pH 7.4. Under a dissecting microscope, a scalpel was used to detach the polyester mesh supporting the organotypic culture from the plastic insert. Organotypic cultures with the polyester mesh support were cut with a scalpel into approximately 2 × 4 mm pieces, and stored overnight in 0.1 M cacodylate buffer, pH 7.4. Following postfixation with 1% osmium tetroxide at 4°C, the organotypic cultures were washed four times, 15 min each, with 0.1 M maleic acid, pH 6.5, before *en bloc* staining with 2% aqueous uranyl acetate for 1 h. After washing with distilled water, organotypic cultures were dehydrated with increasing concentrations of ethanol, followed by 100% propylene oxide, and finally infiltrated with 1:1 propylene oxide/Eponate overnight. Samples were embedded in fresh Eponate in flat embedding molds and oriented so they could be sectioned perpendicularly to the mesh support on a Reichert Ultracut E3 ultramicrotome equipped with a diamond knife. In this way stratified organotypic cultures of keratinocytes attached to the collagen gel or directly to the polyester mesh could be analyzed. Thin sections were stained with lead citrate and examined in a Hitachi H-7000 electron microscope (Hitachi, San Jose, CA) operated at 75 kV.

RESULTS

Isolation of the NIKS cell line Cells were disaggregated by trypsinization of a neonatal foreskin labeled BC-1. Keratinocytes were initiated into culture by plating an aliquot of the cell suspension onto a mitomycin C-treated Swiss mouse 3T3 feeder layer in standard keratinocyte growth medium containing 0.66 mM calcium. Fibroblasts were initiated into culture by plating an aliquot of cell suspension on to a tissue culture plate containing Ham's F-12 medium supplemented with 10% FBS. After approximately 9 d, primary cultures of keratinocytes, designated strain BC-1-Ep, were cryopreserved and subcultured onto a feeder layer. Fibroblast cultures, designated BC-1-F, were grown to near confluence and also cryopreserved. In early passages, the BC-1-Ep cells exhibited no morphologic or growth characteristics that were atypical for cultured normal human keratinocytes. Cultivated BC-1-Ep cells exhibited stratification as well as features of programmed cell death. To determine replicative lifespan, the BC-1-Ep cells were serially cultivated to senescence in standard keratinocyte growth medium at a density of 3×10^5 cells per 100 mm dish and passaged at weekly intervals (approximately a 1:25 split). By passage 15 most keratinocytes in the population appeared senescent as judged by the presence of numerous abortive colonies which exhibited large, flat cells; however, at passage 16, keratinocytes exhibiting a small cell size were evident. By passage 17, only the small sized keratinocytes were present in the culture and no large, senescent keratinocytes were evident (**Fig 1**). The resulting population of small keratinocytes that survived this putative crisis period appeared morphologically uniform and produced colonies of keratinocytes exhibiting typical keratinocyte characteristics including cell–cell adhesion and apparent squame production. The keratinocytes that survived senescence were serially cultivated at a density of 3×10^5 cells per 100 mm dish. Typically the cultures reached a cell density of approximately 8×10^6 cells within 7 d. This stable rate of cell

growth was maintained through at least 59 passages, demonstrating that the cells had achieved immortality. The keratinocytes that emerged from the original senescencing population were originally designated BC-1-Ep/Spontaneous Line (BC-1-Ep/SL) and are now termed NIKS. The NIKS cell line has been screened for the presence of proviral DNA sequences for HIV-1 as well as HPV-16 and HPV-31 using either PCR or Southern analysis. None of these viruses were detected (data not shown).

Cytogenetic analysis and DNA fingerprinting Chromosomal analysis was performed on the parental BC-1-Ep cells at passage 3 and NIKS cells at passages 31 and 54. The parental BC-1-Ep cells have a normal chromosomal complement of 46,XY (**Fig 2*a***). At passage 31, all NIKS cells contained 47 chromosomes with an extra isochromosome of the long arm of chromosome 8 (**Fig 2*b***). No other gross chromosomal abnormalities or marker chromosomes were detected. At passage 54 all cells contained the isochromosome 8; however, additional changes were seen in three cells consisting of an isochromosome of the long arm of chromosome 1 and/or a tiny marker chromosome (**Table I**). Despite some coiling differences between the homologous chromosomes evident in the karyotype in **Fig 2*b***, a band-by-band analysis of each chromosome showed that the i(8q) isochromosome was the only consistent karyotypic abnormality. Although two cells with i(1q) were observed at passage 54, the majority of NIKS cells had the same karyotype seen earlier; cells with the i(1q) or the tiny extra marker, which might reflect transitory changes, still demonstrated the i(8q) isochromosome as the only other chromosomal abnormality. In an effort to determine when the acquisition of the i(8q) isochromosome occurred, we conducted FISH analysis. When 500 interphase parental BC-1-Ep cells (passage 3) were studied using FISH as described (Han *et al*, 1994), the i(8q) was not detected. Evaluation of 100 post-crisis cells (passage 18) with FISH probes for chromosomes 1 and 8 demonstrated extra chromosome 8 signals in approximately half of the cells, but no extra chromosome 1 signals (data not shown). Therefore, the i(8q) isochromosome was present by passage 18.

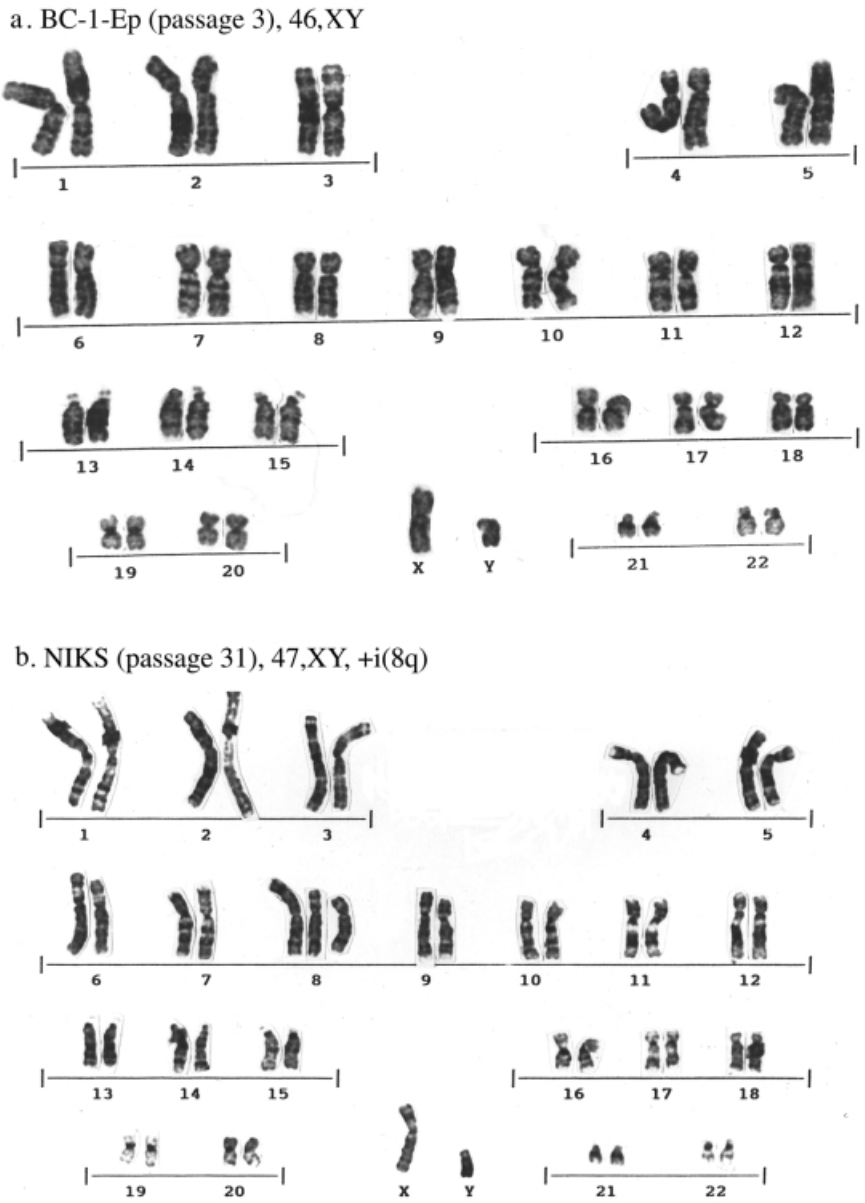

Figure 2. Chromosomal analysis of NIKS cells. Karyotypic analysis was performed on NIKS cells at passage 31. The cells contain 47 chromosomes with an extra isochromosome of the long arm of chromosome 8. The extra chromosome, i(8q), is not seen in the parental keratinocytes (BC-1-Ep, passage 3) which exhibit a normal male karyotype.

The DNA fingerprinting for the NIKS cell line and the BC-1-Ep keratinocytes are identical at all twelve loci analyzed (**Fig 3**). The odds of the NIKS cell line having the parental BC-1-Ep DNA fingerprint by random chance is 4×10^{-16}. The DNA fingerprints from three different sources of human keratinocytes, ED-1-Ep, SCC4, and SCC13y are different from the BC-1-Ep pattern. The data from our DNA fingerprint analysis of the NIKS cell line proves it arose from the parental BC-1-Ep cells. These data also show that keratinocytes isolated from other humans, ED-1-Ep, SCC4, and SCC13y, are unrelated to the BC-1-Ep cells or each other. The NIKS DNA fingerprint data provide an unequivocal way to identify the NIKS cell line.

p53 sequence in the NIKS cell line is wild type Loss of p53 function is associated with an enhanced proliferative potential and increased frequency of immortality in cultured cells (Tsukada *et al*, 1993). For this reason, we investigated the p53 status of the NIKS keratinocytes. The sequence of p53 in the NIKS cells is identical to published p53 sequences (GenBank accession number M14695). In humans, p53 exists in two predominant polymorphic forms distinguished by the amino acid at codon 72. Both alleles of p53 in the NIKS cells are wild type and have the sequence CGC at codon 72, which codes for an arginine (**Fig 4**). The other common form of p53 has a proline at this position. The entire sequence of p53 in the NIKS cells is identical to the BC-1-Ep progenitor cells.

NIKS keratinocytes do not exhibit anchorage-independent growth and are not tumorigenic in athymic nude mice Anchorage-independent growth is highly correlated to tumorigenicity *in vivo* (Shin *et al*, 1975; Ruoslahti and Reed, 1994). For this reason, we investigated the anchorage-independent growth characteristics of NIKS cells in agar or methylcellulose-containing medium. After 4 wk in either agar- or methylcellulose-containing medium, NIKS cells remained single cells. The assays were continued for a total of 8 wk to detect slow growing variants of the NIKS cells. None were observed (data not shown). As a

Table I. Chromosomal aberrations with passage

Passage no.	Spreads	46,XY	47,XY + i(8q)	48,XY + i(1q) + i(8q)	48,XY + i(8q) + mar	49,XY + i(1q) + i(8q) + mar
3[a]	20	20	0	0	0	0
31[b]	20	0	20	0	0	0
54[b]	20	0	17	1	1	1

[a] Parental BC-1-Ep at passage 3.
[b] NIKS at passage 31 and 54.

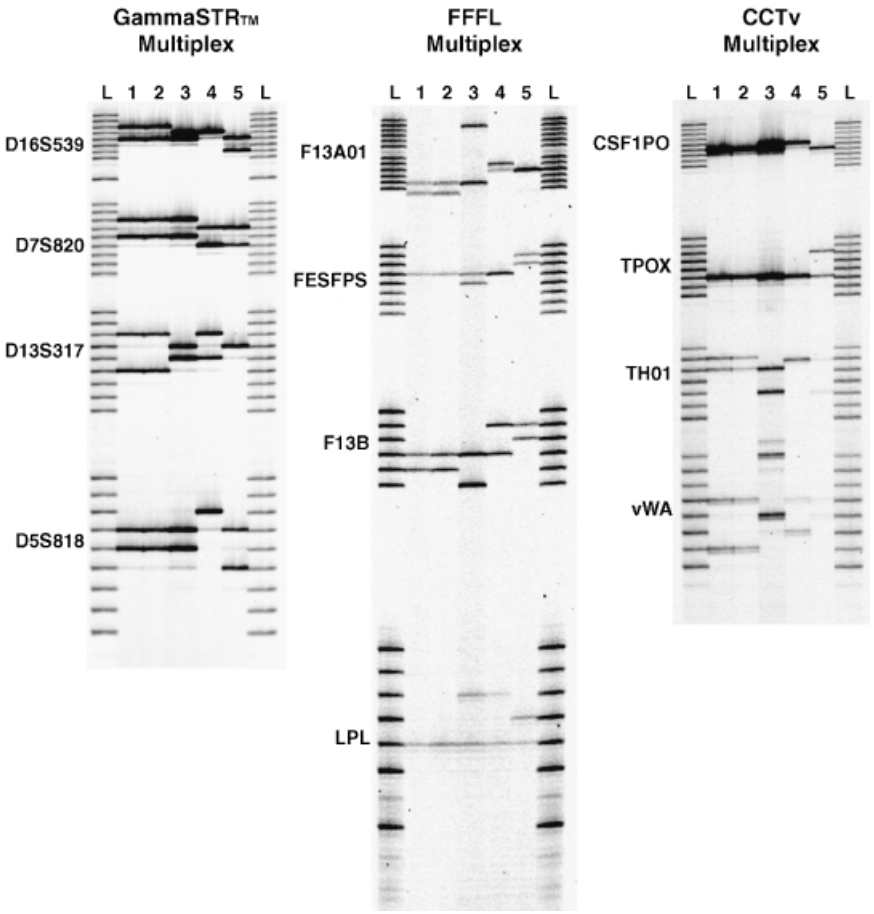

Figure 3. DNA fingerprinting of BC-1-Ep and NIKS cells. Results of a DNA fingerprint gel using a Hitachi FMBIO II Fluorescent Scanner. The 12 primer pairs are divided into three quadriplexes (GammaSTR, FFFL, and CTTv). Each quadriplex tests four independent loci. Individual quadriplexes were amplified in separate reactions using 25 ng of DNA as template. Allelic ladders flank samples (L). BC-1-Ep (*lane 1*) and NIKS (*lane 2*) have identical patterns at all 12 loci. ED-1-Ep (*lane 3*), SCC4 (*lane 4*), and SSC13y (*lane 5*) all have unique patterns.

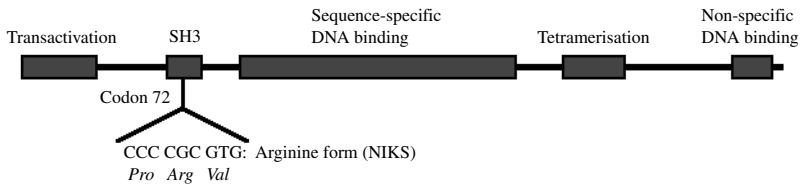

Figure 4. NIKS keratinocytes possess a wild-type p53 with the arginine polymorphism. The structure of the p53 gene is shown illustrating the functional domains of the protein. BC-1-Ep and NIKS cells exhibit a polymorphism at codon 72 resulting in the arginine form of the p53 protein. P53 cDNAs were generated using reverse transcription–PCR on total RNA isolated from the BC-1-Ep and NIKS cells. The sequence of the coding region of p53 was determined by direct sequencing of reverse transcription–PCR products.

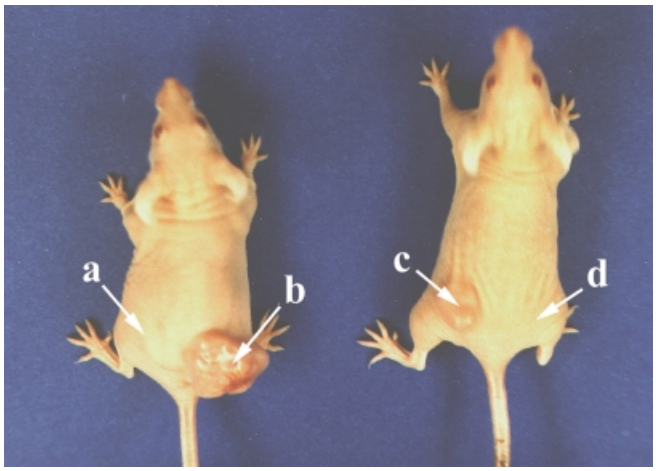

Figure 5. Tumorigenicity of NIKS cells in athymic nude mice. Mice were assessed 31 d after subcutaneous injection of SCC4, BC-1-Ep, and NIKS cells. BC-1-Ep keratinocytes (passage 6) (*a*) and NIKS keratinocytes (passage 35) (*d*) did not form tumors; however, SCC4 cells were tumorigenic (*b*,*c*).

Table II. NIKS do not form tumors in nude mice

Animal no.	Condition	Body weight (g)	Area of tumor (mm^3)
1	left flank = BC-1-Ep6°	22	–
	right = NIKS 35°		–
2	left = BC-1-Ep 6°	24	–
	right = NIKS 35°		–
3	left = SCC4y 20°	21	105.2
	right = NIKS 35°		–
4	left = BC-1-Ep 6°	25	–
	right = SCC4y 20°		1183.6
5	left flank = BC-1-Ep 6°	23	–
	right = NIKS 35°		–
6	left = BC-1-Ep 6°	22	–
	right = NIKS 35°		–
7	left = SCC4y 20°	22	51.3
	right = NIKS 35°		–
8	left = BC-1-Ep 6°	19	–
	right = SCC4y 20°		463.3

positive control, NRK-49F cells were used to test the ability of the agar and methylcellulose media to support anchorage-independent growth. The media used to test anchorage-independent growth of NIKS cells supported TGF-β1-mediated anchorage-independent growth of NRK-49F cells (data not shown).

To determine the tumorigenicity of the parental BC-1-Ep keratinocytes and the immortal NIKS keratinocyte cell line, cells were injected into the flanks of athymic nude mice. The human squamous cell carcinoma cell line, SCC4, was used as a positive control for tumor production in athymic nude mice. The injection of samples was designed such that animals received an injection of SCC4 cells in one flank and either the parental BC-1-Ep keratinocytes or the NIKS cells in the opposite flank. This injection strategy eliminated animal to animal variation in tumor production and confirmed that the mice would support vigorous growth of tumorigenic cells. Neither the parental BC-1-Ep keratinocytes (passage 6) nor the NIKS keratinocytes (passage 35) produced tumors in athymic nude mice (**Fig 5**). The results of the tumorigenicity testing are shown in **Table II**.

Growth characteristics *in vitro* NIKS keratinocytes exhibit morphologic characteristics of normal human keratinocytes when cultured in standard keratinocyte growth medium in the presence of mitomycin C-treated 3T3 feeder cells. To evaluate further the growth characteristics of the NIKS cells, we examined the steady-state mRNA levels of known autocrine regulators of keratinocyte growth. Northern analysis of mRNAs from the NIKS cell line revealed that expression of genes encoding autocrine growth factors, such as TGF-α and TGF-β1, as well as the levels of epidermal growth factor receptor (EGFR) and c-*myc*, are similar, if not identical to the parental BC-1-Ep keratinocytes (**Fig 6**). Both the parental cells and the NIKS cells express identical mRNA levels of the basal keratinocyte-specific keratin, K14.

We next determined which constituents of standard keratinocyte growth medium are required for optimal growth of NIKS cells. Serial cultivation in the absence of EGF resulting in a 60–90% reduction in cell number at each passage, compared with EGF-containing control cultures (**Fig 7**). The dependence on EGF for growth of NIKS cells appears to be a stable characteristic. NIKS cells at passage 50 continue to exhibit a dependence on EGF for optimal growth (data not shown). Another polypeptide growth factor that plays an important part in epidermal homeostasis is TGF-β1. *In vitro*, TGF-β1 is an inhibitor of growth in cultured normal human keratinocytes (Shipley *et al*, 1986; Pietenpol *et al*, 1990a); however, malignant transformation of keratinocytes often results in attenuation of TGF-β1-induced growth inhibition (Bascom *et al*, 1989; Pietenpol *et al*, 1990b; Krieg *et al*, 1991). Like the normal parental BC-1-Ep keratinocytes, TGF-β1 inhibits the growth of the NIKS cell line (**Fig 8**). The TGF-β1-induced growth inhibition is reversible in both the parental and NIKS keratinocytes (data not shown).

To further characterize the requirements for optimal *in vitro* growth of the NIKS keratinocytes, cells were cultivated in medium supplemented with 2.5% FBS, EGF, and individual constituents of the standard growth medium. **Figure 9** demonstrates that addition of insulin alone promotes a 15-fold increase in cell number. Addition of all constituents of standard keratinocyte growth medium, however, promotes a 30-fold increase in NIKS cell number. These findings demonstrate that the NIKS cell line has maintained cell-type-specific requirements for growth *in vitro*.

Differentiation characteristics *in vitro* NIKS cells were analyzed for the ability to undergo differentiation in both surface culture and organotypic culture. For cells in surface culture, we monitored a marker of squamous differentiation, the formation of

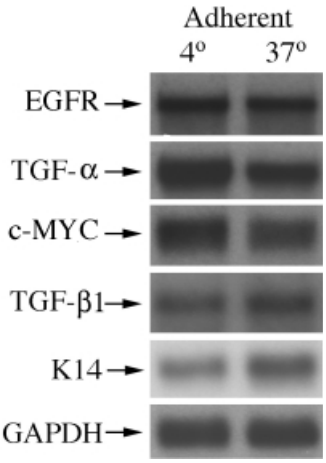

Figure 6. Steady-state mRNA levels of growth regulatory and cytoskeletal proteins in parental and NIKS keratinocytes. Steady-state mRNA levels for EGFR, TGF-α, c-*myc*, TGF-β1, and keratin 14 (K14) were analyzed in BC-1-Ep (passage 4) and NIKS (passage 37). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Each lane contains 3 μg of poly(A)$^+$ RNA. Northern analysis was performed using random primer-labeled [^{32}P]cDNA probes.

CE. In cultured human keratinocytes, early stages of CE assembly result in the formation of an immature CE composed of involucrin, cystatin-α and other proteins, which represent the innermost third of the mature CE. We examined CE formation in the parental and the NIKS keratinocytes (**Table III**). Less than 2% of the keratinocytes from either the adherent BC-1-Ep cells or the NIKS cell line produce CE. This finding is consistent with our previous studies demonstrating that actively growing, subconfluent keratinocytes produce less than 5% CE (Hines and Allen-Hoffmann, 1996b). To determine whether the NIKS cell line is capable of producing CE when induced to differentiate, the cells were removed from surface culture and placed in suspension for 24 h in medium made semi-solid with methylcellulose. Many aspects of terminal differentiation, including differential expression of keratins (Drozdoff and Pledger, 1993) and CE formation (Green, 1977) can be triggered *in vitro* by loss of keratinocyte cell–cell and cell–substratum adhesion. We found that the NIKS keratinocytes produced as many as and usually more CE than the parental keratinocytes (**Table III**). These findings demonstrate that the keratinocytes are not defective in their ability to initiate the formation of this cell-type-specific differentiation structure.

NIKS cells undergo nucleosomal fragmentation following loss of adhesion We next determined if NIKS keratinocytes exhibit nucleosomal cleavage of DNA. Specific DNA cleavage into oligonucleosomal fragments is a hallmark of apoptosis (reviewed in Wyllie, 1980; Loo and Rillema, 1998). Keratinocytes from all species studied to date can undergo apoptosis both *in vivo* and *in vitro* (reviewed in Polakowska *et al*, 1994; Haake *et al*, 1998). Epidermal keratinocytes are destined to enucleate and lose metabolic activity as part of their differentiation pathway. Many parallels exist between keratinocyte terminal differentiation and apoptosis. We assessed the ability of the parental and NIKS keratinocytes to undergo nucleosomal cleavage following suspension (**Fig 10**). Normal cultured human keratinocytes exhibit both morphologic and biochemical features of apoptosis when deprived of cell–cell and cell–substrata contact by suspension in semi-solid medium (Hines and Allen-Hoffmann, 1996b; Sachsenmeier *et al*, 1996; Gandarillas *et al*, 1999). Adherent, preconfluent keratinocytes from parental or NIKS cells grown in serum-free, additive-free medium for 24 h exhibited no detectable DNA fragmentation (**Fig 10**, *lanes 1* and *4*). Similarly, adherent cells treated for an identical time with semi-solid, serum-free, additive-free medium did not exhibit nucleosomal fragmentation (**Fig 10**, *lanes 2* and *5*). When suspended in semi-solid medium both the BC-1-Ep and NIKS keratinocytes exhibited DNA fragmentation (**Fig 10**, *lanes 3* and *6*). These findings are consistent with previous studies from our laboratory demonstrating that normal human keratinocytes induced to differentiate by loss of adhesion will fragment their DNA (Hines and Allen-Hoffmann, 1996a,b; Sachsenmeier *et al*, 1996). Taken together, these data demonstrate that the NIKS keratinocytes are capable of differentiating and respond normally to cell-type-specific signals to undergo nucleosomal fragmentation.

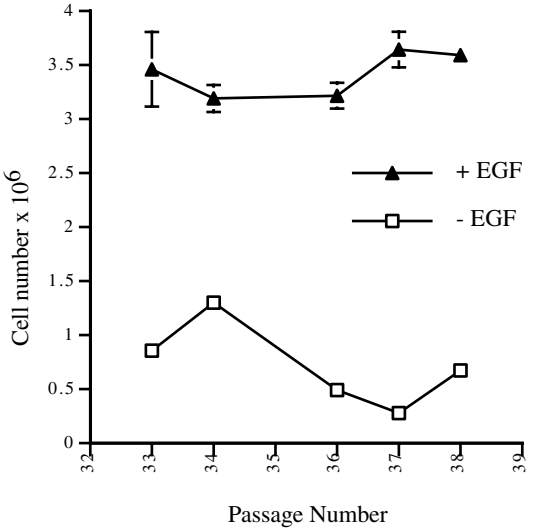

Figure 7. NIKS cells require EGF for optimal growth. NIKS keratinocytes were plated at 1×10^5 cells per 60 mm dish on a 3T3 feeder layer in standard keratinocyte growth media with and without 10 ng EGF per ml. Triplicate cultures were counted and subcultured every 7 d to determine the effect of EGF on serial cultivation of NIKS keratinocytes. Values represent the mean of triplicate samples with standard deviations indicated by the bar. Error bars in EGF data are smaller than the data points.

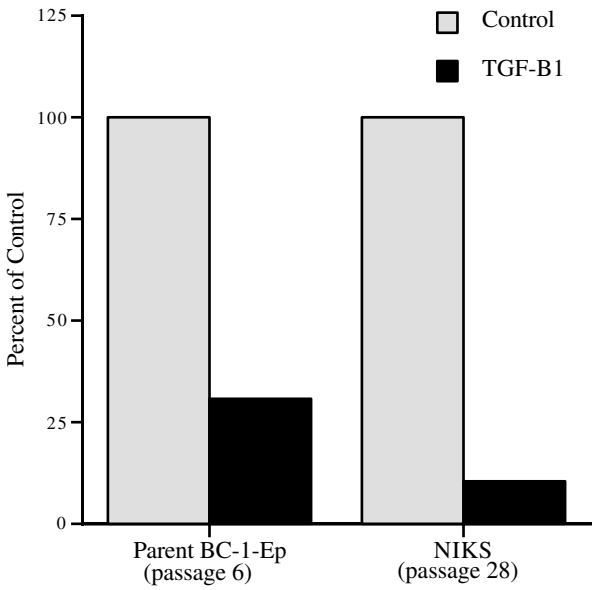

Figure 8. TGF-β1 inhibits growth of NIKS keratinocytes. The parent cells, BC-1-Ep (passage 6), and NIKS (passage 28) were plated onto six-well cluster dishes in standard keratinocyte growth media without EGF in the absence of a 3T3 feeder layer. When cultures were approximately 20% confluent, triplicate dishes were treated with TGF-β1 (5 ng per ml) and counted 3–5 d later. The effect of TGF-β1 on cell number is expressed as a percentage of controls.

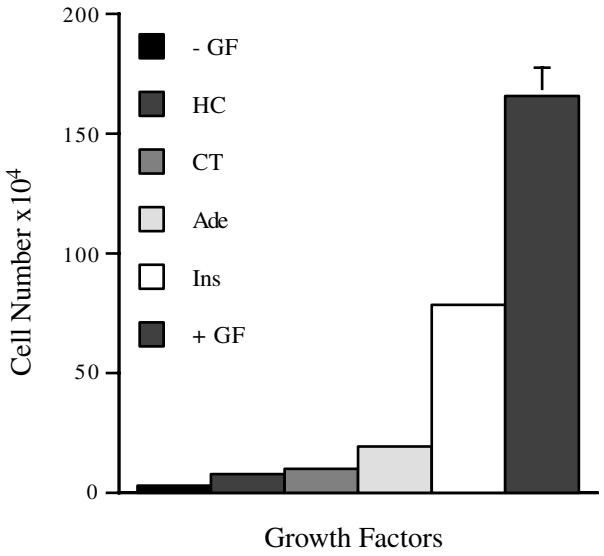

Figure 9. Growth factor requirements of NIKS cells. NIKS cells (passage 31) were plated at a density of 5×10^4 in six-well cluster dishes containing a 3T3 feeder layer and basal growth medium composed of Ham's F-12/DME (3:1, final calcium concentration 0.66 mM) supplemented with 2.5% FBS, 10 ng per ml EGF, P/S, and no additional additives (–GF). The basal growth medium was supplemented with the following: 0.4 μg per ml HC, 8.4 ng CT per ml, 24 μg Ade per ml, 5 μg Ins per ml, or all growth factors (+GF). Cells were maintained in the presence of each medium condition for 8 d. Values represent the mean of four samples with standard deviations indicated by the bar.

Organotypic cultures of NIKS cells exhibit squamous differentiation To demonstrate that the NIKS keratinocytes can undergo squamous differentiation, the cells were cultivated in organotypic culture. Keratinocyte cultures grown on plastic substrata and submerged in medium replicate but exhibit limited differentiation. Specifically, human keratinocytes become confluent and undergo limited stratification producing a sheet consisting of three or more layers of keratinocytes. By light and electron microscopy there are striking differences between the architecture of the multilayered sheets formed in tissue culture and intact human skin (reviewed in Fusenig, 1994). Organotypic culturing techniques allow for keratinocyte growth and differentiation under *in vivo*-like conditions. Specifically, the cells adhere to a physiologic substratum consisting of dermal fibroblasts embedded within a fibrillar collagen base. The organotypic culture is maintained at the air–medium interface. In this way, cells in the upper sheets are air-exposed whereas the proliferating basal cells remain closest to the gradient of nutrients provided by diffusion through the collagen gel. Under these conditions, correct tissue architecture is formed.

Morphologic features of the parental cells, BC-1-Ep, and the cell line, NIKS, grown in organotypic culture are shown in **Fig 11**. Several characteristics of a normal differentiating epidermis are evident. In both the parental cells and the NIKS cell line a single layer of cuboidal basal cells rests at the junction of the epidermis and the dermal equivalent. The rounded morphology and high nuclear to cytoplasmic ratio is indicative of an actively dividing population of keratinocytes. In normal human epidermis, as the basal cells divide they give rise to daughter cells that migrate upwards into the differentiating layers of the tissue. The daughter cells increase in size and become flattened and squamous. Eventually these cells enucleate and form cornified, keratinized structures. This normal differentiation process is evident in the upper layers of both the BC-1-Ep parental cells and the NIKS cells (**Fig 11*a*, *b***). The appearance of flattened squamous cells is evident in the upper layers of keratinocytes located above the basal layer and demonstrates that stratification has occurred in the organotypic cultures. In the uppermost part of the organotypic cultures (a and b), the enucleated squames are shown peeling off the top of the culture. To date, we have not observed any histologic differences in differentiation at the light microscope level between the parental BC-1-Ep keratinocytes and the NIKS keratinocyte cell line grown in organotypic culture.

To observe more detailed characteristics of the BC-1-Ep (passage 5) and NIKS (passage 38) organotypic cultures and to confirm our histologic observations, samples were analyzed using electron microscopy. Parental BC-1-Ep cells and the immortalized human keratinocyte cell line NIKS were harvested after 15 d in organotypic culture and sectioned perpendicularly to the basal layer to show the extent of stratification. Both the parental BC-1-Ep cells and the NIKS cell line undergo extensive stratification in organotypic culture (**Fig 12*A*, *B***). Abundant desmosomes are formed in organotypic cultures of parental BC-1-Ep cells and the NIKS cell line. We also noted the formation of a basal lamina and associated hemidesmosomes in the basal keratinocyte layers of both the parental cells and the cell line. This was especially evident in

Table III. Formation of cornified envelopes in NIKS

	Treatment	Adherent	Suspended
Exp. 1	Parent (4°)	1.51%	43.3%
	NIKS (31°)	1.32%	69.8%
Exp. 2	Parent (6°)	1.79%	32.8%
	NIKS (33°)	1.05%	73.7%

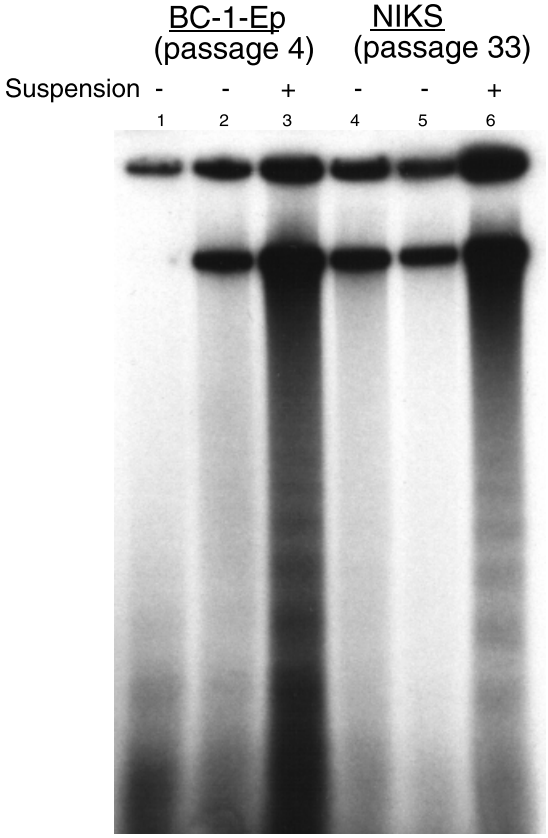

Figure 10. NIKS cells exhibit nucleosomal fragmentation. Genomic DNA was isolated from adherent BC-1-Ep (passage 4) (*lanes 1–3*) and NIKS (passage 33) (*lanes 4–6*) treated 24 h with serum-free basal growth medium (*lanes 1* and *4*), overlaid with (*lanes 2* and *5*), or suspended in methylcellulose-containing serum-free basal medium (*lanes 3* and *6*). Following treatment, genomic DNA was isolated, labeled on the 3′ ends by [α-^{32}P]ddATP, electrophoresed through a 1.5% agarose gel and visualized by autoradiography.

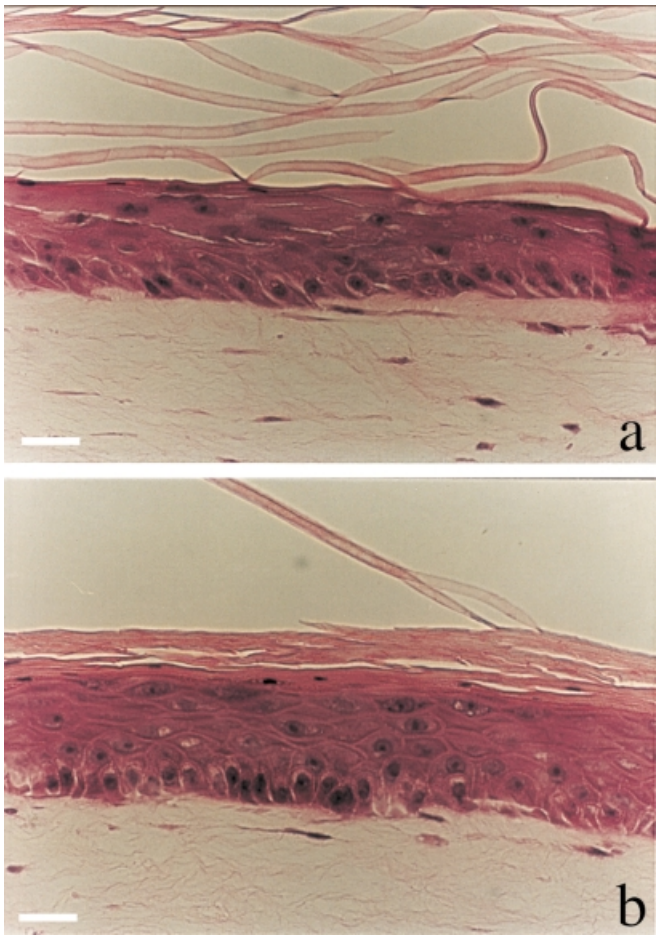

Figure 11. Histologic sections of the parental BC-1-Ep keratinocytes and the NIKS keratinocyte cell line in organotypic culture. BC-1-Ep (passage 5) (*a*) and NIKS keratinocytes (passage 38) (*b*) grown in organotypic culture. Four days after plating, the cells were lifted to the air–liquid interface to induce further differentiation. After 11 d at the air–liquid interface, half of each raft was fixed, sectioned, and stained with hematoxylin and eosin. Sections were viewed and photographed at the light microscope level. *Scale bar*: 30 μm.

areas where the parental cells or the NIKS cells had attached directly to the mesh support (**Fig 12*C*, *D***). Our findings are consistent with earlier ultrastructural findings by Contard *et al.* (1993) using human foreskin keratinocytes cultured on a fibroblast-containing nylon mesh. Hemidesmosomes are specialized structures that increase adhesion of the keratinocytes to the basal lamina and help maintain the integrity and strength of the tissue. Analysis at both the light and electron microscopic levels demonstrate that the NIKS cell line in organotypic culture can stratify, differentiate, and form structures such as desmosomes, basal lamina, and hemidesmosomes found in normal human epidermis.

DISCUSSION

We report the isolation of a spontaneously immortalized human keratinocyte cell line, NIKS. The cell line arose from a culture of neonatal foreskin keratinocytes isolated from an apparently normal male infant. The early passage BC-1-Ep keratinocytes possess a normal male karyotype. Following passage 15, however, a new population of small, homogeneous cells was visually evident in a single culture of senescing keratinocytes. The ability to continuously serial subcultivate these post-crisis keratinocytes in standard keratinocyte growth medium suggests that they had achieved immortality. The keratinocyte cell line was named NIKS indicating that the cells are a spontaneously immortalized line originated from a single keratinocyte strain. DNA fingerprinting results confirm that the NIKS cells originated from the parental BC-1-Ep keratinocytes. Karyotypic analysis shows that by passage 30 all of the cultured NIKS cells contain 47 chromosomes with an extra isochromosome of the long arm of chromosome 8. The near diploid karyotype appears to be a stable characteristic of the NIKS keratinocytes. The cell line exhibits cell-type-specific requirements for monolayer growth *in vitro*. NIKS keratinocytes grow optimally when cultured under conditions identical to those of diploid normal human keratinocytes. Furthermore, the differentiation program of NIKS keratinocytes appears to be intact, making the cell line a particularly valuable cellular reagent for the study of human keratinocyte growth and differentiation.

Spontaneous immortalization of normal human keratinocytes is a rare occurrence. The NIKS keratinocyte line exhibits a single chromosome change as an early characteristic immediately following senescence of the parental population. The presence of two extra copies of the chromosome 8 long-arm in the isochromosome could contribute to the growth advantage of cultured NIKS keratinocytes. For example, the human c-*myc* gene is located on the long arm of chromosome 8 (Neel *et al*, 1982; Taub *et al*, 1982); however, NIKS keratinocytes do not possess increased steady-state levels of c-*myc* mRNA. Trisomy 8 is frequently associated with hyperproliferation in human cells. Many forms of human leukemia and lymphoma exhibit trisomy at chromosome 8 along with other chromosomal aberrations (Mitelman, 1991). Trisomy 8 is also found in other neoplasms such as breast cancer (Pandis *et al*, 1993) and prostate carcinoma (Macoska *et al*, 1993). The NM 1 human keratinocyte cell line isolated and characterized by Baden *et al.* (1987) exhibited trisomy of chromosome 8. The NM 1 cell line gave rise to a stable clone with 46 chromosomes in which two of the three number 8 chromosomes were replaced by an i(8q) isochromosome, thereby retaining three copies of the chromosome 8 long-arm with monosomy of the short-arm (Goldaber *et al*, 1990). Although the stability of this chromosomal aberration and the acquisition of additional abnormalities at later passages have not been investigated, it is of interest that either an extra chromosome 8 or an extra copy of the chromosome 8 long-arm was associated with immortalization. As the NM 1 cell line arose from cells cultured from pooled human neonatal foreskins, it is impossible to compare chromosomal and biochemical characteristics of the immortalized cells with the parental populations. Rice *et al.* (1993) reported the isolation of a spontaneously immortalized keratinocyte line, SIK, from a single neonatal foreskin. Unlike the parental BC-1-Ep keratinocytes, mass cultures of the early passage parental keratinocytes from which the SIK cells were derived gave rise to other keratinocyte populations with vigorous growth characteristics. This finding suggests that the parental population was genetically predisposed to exhibit enhanced growth. By passage 20 the majority of SIK cells had acquired an isochromosome, i(6p), in addition to other low-frequency chromosomal abnormalities. An additional isochromosome, i(8q), is present in all SIK cells by passage 78. Similarly, Weaver *et al.* (1991) reported that partial trisomy of cultured human keratinocytes at chromosomes 9 and 18 are characteristic of the long-lived keratinocyte lines HKC-N2 and HKC-N6, which arose from a commercial source of normal human keratinocytes. Finally, the HaCaT cell line, generated from adult skin located at the distant periphery of a malignant melanoma, exhibited distinct cytogenetic abnormalities after being subcultured twice in 8 mo in low calcium medium and cultured at an elevated temperature of 38.5°C (Boukamp *et al*, 1988; Fusenig and Boukamp, 1998). Most passage 2 HaCaT keratinocytes exhibited a hypodiploid karyotype with 44 chromosomes resulting from the loss of the Y chromosome and partial monosomies due to centric fusions and isochromosome formation; however, trisomy of chromosomes 6 or 8 is not observed at this early passage. HaCaT keratinocytes harbor numerous marker chromosomes, especially after passage 5 when a hypertetraploid subclone(s) evolved. At this point, trisomy of chromosome 6 and tetrasomy of chromosome 8

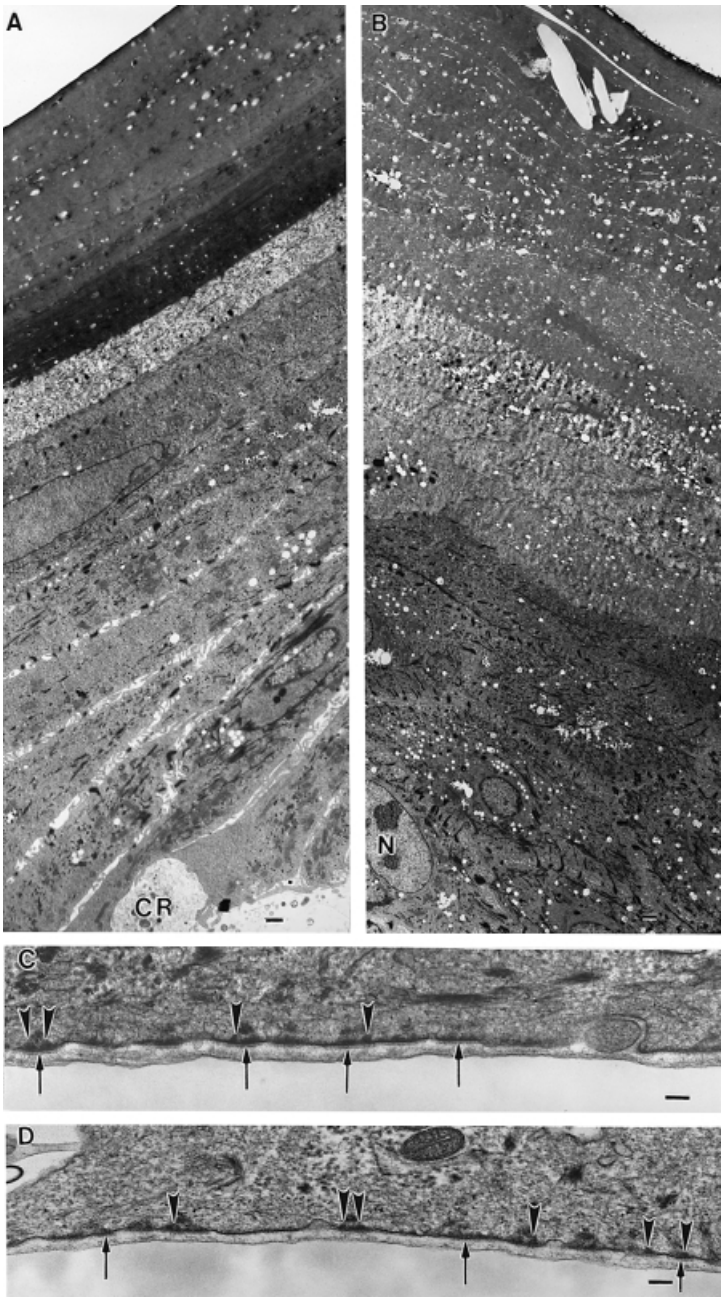

Figure 12. Ultrastructure of the parental BC-1-Ep keratinocytes and the NIKS keratinocyte cell line in organotypic culture exhibit stratification and differentiation. Organotypic cultures of parental BC-1-Ep (passage 5) (*A*) and the immortalized NIKS keratinocytes (passage 38) (*B*) were sectioned perpendicularly to illustrate basal keratinocytes with nuclei (N) attached to the collagen raft (CR) substrate. Both parental and cell line cultures exhibit extensive stratification, resulting in daughter cells that enucleate and undergo terminal differentiation. Basal keratinocytes which are attached to the polyester mesh supporting the collagen raft in both the parental BC-1-Ep (*C*) and the cell line NIKS (*D*) form basal laminae (*arrows*) and numerous hemidesmosomes (*arrowheads*). *Scale bars*: (*A, B*) 1 μm; (*C, D*) 0.1 μm.

were observed in the HaCaT keratinocytes. We found that NIKS keratinocytes exhibit a supernumerary i(8q), but no other consistent chromosomal imbalance. Our finding that i(q8) is a consistent, stable characteristic of the spontaneously immortal NIKS cells suggests that extra copies of genes on the long arm of chromosome 8 may be sufficient for immortalization of human keratinocytes.

Historically, cytogenetic analysis of immortalized cells has been used as a way to categorize gross alterations at the chromosomal level. Specific genes involved in conferring immortality, however, cannot be definitively identified by this approach. Individual genes have been identified that contribute to loss of growth control during neoplastic development in mammalian cells. Mutation of the p53 gene is associated with over 50% of all human cancers. In normal cells, the wild-type p53 gene product orchestrates cellular responses to stress (reviewed in Levine, 1997). The best-characterized p53-mediated cellular responses to stress include cell cycle arrest and apoptosis. Wild-type p53 has also been implicated in cellular senescence. Cultured cells isolated from p53 nullizygous mice produce long-lived and immortalized cell lines at a high frequency (Harvey *et al*, 1993; Tsukada *et al*, 1993; Lowe *et al*, 1994). For this reason, we investigated the p53 status of the NIKS cell line. In humans, a sequence polymorphism is observed at codon 72 resulting in either proline (p53pro) or arginine (p53arg) forms of the wild-type protein (Matlashewski *et al*, 1987). The allelic frequency of the arginine form of p53 differs significantly between human racial groups (Beckman *et al*, 1994). We found that the NIKS keratinocytes possess a wild-type p53 with the arginine polymorphism. It has been suggested that susceptibility of cells to viral, specifically human papilloma virus, and other transforming agents may be related to the p53 allele they contain (Storey *et al*, 1998). Additional studies are required to determine whether the different human p53 polymorphisms contribute to cellular susceptibility to oncogenic agents. Unfortunately, the most widely studied human keratinocyte cell line, HaCaT cells, exhibit mutations in both alleles of the p53 gene (Lehman *et al*, 1993). The presence of a wild-type p53 in the NIKS keratinocytes makes it an attractive cellular model to test the putative role of the arginine polymorphism in oncogenic susceptibility.

The NIKS cell line maintains cell-type-specific growth and differentiation characteristics in monolayer and organotypic culture. We found that NIKS cells are responsive to growth factors such as EGF and TGF-β1, which are known to regulate keratinocyte growth. This is in contrast to HaCaT cells which at later passages exhibit anchorage independent growth, have high colony-forming efficiency, reach high saturation densities, and do not require cell-type-specific culture conditions for serial cultivation (Fusenig and Boukamp, 1998). Unlike other spontaneously immortalized keratinocyte cell lines such as HaCaT (Boelsma *et al*, 1999; Schoop *et al*, 1999), NIKS keratinocytes differentiate to the same extent and at the same rate as the parental BC-1-Ep keratinocytes when cultivated in organotypic culture with dermal fibroblasts. Organotypic culturing is a valuable tool to assess squamous differentiation of human keratinocytes. The NIKS cells form a stratified epithelium histologically identical to the parental cells and other normal keratinocyte strains. The multilayered keratinizing epithelium is highly organized and exhibits features typical of intact skin such as hemidesmosomes, desmosomes, keratin tonofilaments, and keratohyalin granules. Smola *et al*. (1998) have clearly shown that organotypic cultures composed of normal dermal fibroblasts and keratinocytes develop a basement membrane zone capable of supporting cell-type-specific adhesion structures such as hemidesmosomes. We found that both the parental BC-1-Ep and NIKS keratinocytes produce hemidesmosomes in organotypic culture suggesting that the synthesis, deposition, and assembly of extracellular matrix glycoproteins has occurred.

In summary, the growth and differentiation characteristics of the NIKS keratinocyte cell line make it an important new model for the study of human epidermal biology. We can readily obtain stable transfection of the cell line (Liliensiek, Schlosser, Ryan, and Allen-Hoffmann, manuscript in preparation). The NIKS keratinocytes have been stably transfected with green fluorescent protein and organotypic cultures of green fluorescent protein-expressing NIKS keratinocytes produce a normal multilayered keratinizing epithelium. The ability to transfect human keratinocytes stably that possess both self-renewing capacity and the ability to differentiate provides exciting new opportunities for mechanistic studies on growth and differentiation of normal and aberrant keratinocytes in human stratified squamous epithelia. For example, we have developed a tumor model system where the NIKS cells serve as a well-defined and consistent source of ostensibly normal human keratinocytes. Human squamous cell carcinoma cells are co-cultured with NIKS cells in an organotypic system to create a tissue-like model of human skin cancer (Pickart, Clotfelter, and Allen-Hoffmann, manuscript in preparation). The NIKS keratinocyte cell line also supports growth of the high-risk human papilloma virus 16 in organotypic culture (Flores *et al*, 1999a,b). The study of human papilloma viruses in cultured normal human keratinocytes is limited to those viruses that extend the lifespan of early passage keratinocytes. Because NIKS keratinocytes are immortal yet undergo squamous differentiation, they may allow researchers to study the viral life cycle of other high- and low-risk human papilloma viruses. The human NIKS keratinocyte cell line represents an important new cellular tool for addressing fundamental questions regarding epithelial cell biology, toxicities, and pathologies.

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