Establishment and Characterization of an Immortalized Human Sebaceous Gland Cell Line (SZ95)[1]

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Human facial sebaceous gland cells were transfected with a PBR-322-based plasmid containing the coding region for the Simian virus-40 large T antigen. The resulting proliferating cell cultures have been passaged over 50 times to date, have been cloned, and show no signs of senescence after 4½ y *in vitro*, whereas normal human sebocytes can only be grown for three to six passages. The immortalized transfected cells, termed SZ95, expressed the Simian virus-40 large T antigen and presented an hyperdiploid–aneuploid karyotype with a modal chromosome number of 64.5. The SZ95 cell line exhibited epithelial, polymorphous characteristics with different cell sizes of up to 3.25-fold during proliferation and 6-fold at confluence, showing numerous cytoplasmic lipid droplets. The cells showed large cytoplasm profiles with abundant organelles, including vacuoles and myelin figures which indicated lipid synthesis. Lack of or only few desmosomal areas were observed. SZ95 cells expressed molecules typically associated with human sebocytes, such as keratins 7, 13, and 19, and several proteins of the polymorphous epithelial mucin family. Functional studies revealed synthesis of the sebaceous lipids squalene and wax esters as well as of triglycerides and free fatty acids, even after 25–40 passages; active lipid secretion; population doubling times of 52.4 ± 1.6 h; reduced growth but maintenance of lipid synthesis under serum-free conditions; and retrieval of cell proliferation after addition of 5α-dihydrotestosterone. Retinoids significantly inhibited proliferation of certain SZ95 cell clones in the expected magnitude 13-*cis*-retinoic acid > all-*trans*-retinoic acid > > acitretin. Thus SZ95 is an immortalized human sebaceous gland cell line that shows the morphologic, phenotypic and functional characteristics of normal human sebocytes. ***Key words: androgens/cell transformation/viral antigens/polyomavirus transforming/retinoids. J Invest Dermatol 113:1011–1020, 1999***

There is increasing evidence that sebocytes may play crucial parts in the pathophysiologic processes and disorders of the pilosebaceous unit, especially in acne (Gollnick *et al*, 1991; Brown and Shalita, 1998; Cunliffe, 1998; Strauss, 1998). To date, much of our understanding of the physiology and pathophysiology of the sebaceous gland stems from experimental animal models (Pochi, 1985) but no animal model was found predictive in assessing the effects of anti-acne drugs in human beings (Shapiro *et al*, 1989; Geiger, 1995). The facts that acne is an exclusively human disease and that the secretory activity of the sebaceous gland is remarkably species-specific (Nikkari, 1974) led to the search for human models.

Initial studies have been performed on whole human skin plugs, either been incubated *in vitro* (Hsia *et al*, 1970; Cooper *et al*, 1976; Sharp *et al*, 1976) or grafted on to nude mice (Petersen *et al*, 1984). Acquisition of significant knowledge on sebocyte function at the cellular level, however, began with the isolation of viable human sebaceous glands (Kealey *et al*, 1986) and the establishment of the human sebocyte culture model *in vitro* (Xia *et al*, 1989). Sebocyte markers have been identified and expression of proteins recognizing sebocyte differentiation have been detected (Zouboulis *et al*, 1991c, 1994). Free fatty acids were shown to be synthesized by sebocytes without bacterial influence (Zouboulis *et al*, 1991a) and were found to play an active part on sebocyte proliferation (Akai *et al*, 1994). The behavior of sebocytes to androgens *in vitro* was found to correlate to the anatomic localization of their origin (Akamatsu *et al*, 1992). Facial sebocytes were shown to exhibit *in vitro* a stronger 5α-reductase expression that other cultured cells derived from adult skin (Chen *et al*, 1998) and their proliferation was stimulated by 5α-dihydrotestosterone (5α-DHT) in a higher magnitude than by testosterone (Akamatsu *et al*, 1992). Cultured sebocytes were also found sensitive to several hormones other than androgens, such as insulin, hydrocortisone, and thyroid-stimulating hormone (Zouboulis *et al*, 1998). Retinoic acid receptors-γ and -α and retinoid X receptor-α were detected in human sebocytes at the mRNA level (Doran *et al*, 1991a). 13-*cis*-Retinoic acid exhibited an independent regulation of proliferation, lipid synthesis, and terminal differentiation of human sebocytes *in vitro* (Zouboulis *et al*, 1991a, 1994), whereas monoaromatic retinoids were barely active (Doran and Shapiro, 1990; Zouboulis *et al*, 1991a). Finally, retinoids were shown to substitute partially the effects of vitamin A on sebocyte activity *in vitro* after vitamin A depletion (Zouboulis *et al*, 1993).

Manuscript received April 7, 1999; revised August 4, 1999; accepted for publication August 17, 1999.

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Abbreviations: 5α-DHT, 5α-dihydrostestosterone; SV, Simian virus.

[1]The authors have declared a conflict of interest.

Table I. Monoclonal antibodies used in immunocytochemical and western blot characterization of SZ95 cells *in vitro*[a]

MoAb	Commercial source	Dilution	Specificity for	Mol. weight	Reference
Immunocytochemistry					
OM-1 (m)	–	1:100	Sebaceous gland antigen		De Kretser (1988)
Western blot analysis					
RCK 105 (m)	ICN, Aurora, OH	1:100	Keratin 7	54 kDa	Ramaekers *et al* (1987)
1C7 (m)	ICN, Aurora, OH	1:100	Keratin 13	54 kDa	Van Muijen *et al* (1986)
RPN1165 (m)	Amersham, Braunschweig, Germany	1:50	Keratin 19	40 kDa	Lane *et al* (1985)
HMFG-1 (m)	Immunotech, Hamburg, Germany	1:100	Human milk fat globulin-1	400 kDa	Taylor-Papadimitriou *et al* (1981)
HMFG-2 (m)	Immunotech, Hamburg, Germany	1:100	Human milk fat globulin-2	80–400 kDa	Taylor-Papadimitriou *et al* (1981)
Anti-ESM (m)	ICN, Aurora, OH	1:100	Human epithelial sialo-mucin, MAM-6	> 400 kDa	Tsubura *et al* (1987)
DAKO-HB-T1 (m)	Dako, Glostrup, Denmark	1:40	Thomsen-Friedenreich antigen	155 kDa	Karsten *et al* (1995); Kanitakis *et al* (1998)
MCA (m)	Immunotech, Hamburg, Germany	1:100	Mucin-like carcinoma-associated antigen	350 kDa	Stähli *et al* (1998)
EMA (m)	Dako, Glostrup, Denmark	1:100	Epithelial membrane antigen	250–400 kDa	Ormerod *et al* (1983); Baumrind *et al* (1992)
5α-Reductase (r)	–	1:4000	5α-Reductase type 1	21–27 kDa	Luu-The *et al* (1994); Chen *et al* (1998)

[a]The expression of a series of anti-keratin MoAb, MoAb against human polymorphous epithelial mucin and a MoAb against 5α-reductase type 1 was screened. All MoAb have been previously described. m, mouse; r, rabbit.

Figure 1. Detection of the SV-40 large T antigen. Cytocentrifuge preparations of (*a*) SZ95 cells and (*b*) HMEC-1 cells positively labeled with a MoAb against the human SV-40 large T antigen. Both cells types mostly reveal strong nuclear staining, whereas some cells also exhibit cytoplasmic labeling. *Scale bar*: 3 μm. (*c*) Western blot analysis of SV-40 large T antigen expression in non-transfected human sebocytes (*lane 1*), human keratinocytes (*lane 2*), SZ95 cells (34th subculture) (*lane 3*), SZ95/K6 cells (*lane 4*), SK95/K7 cells (*lane 5*), and SZ95/K28 cells (*lane 6*). A 94 kDa band, compatible for the SV-40 large T antigen, was detected in the SZ95 cell line and its clones.

Over the last years, modifications of the technique of Xia *et al* (1989) have facilitated reproducible cultivation of human sebocytes *in vitro* (Lee, 1990; Zouboulis *et al*, 1991c, 1993; Akamatsu *et al*, 1992; Fujie *et al*, 1996; Chen *et al*, 1998). Human sebocytes, however, are predestined to differentiate by accumulating neutral fat droplets until they burst and die. Therefore, adequate cell amounts for large-scale experiments can only be obtained from

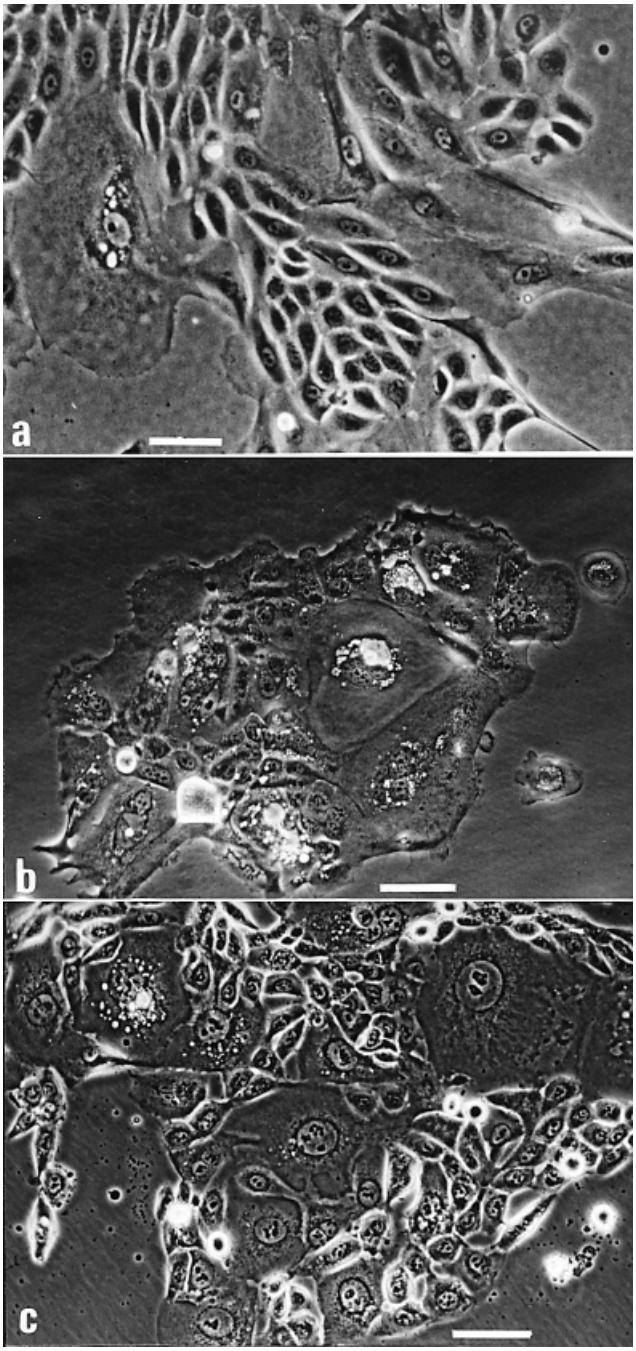

Figure 2. Cell morphology. Subconfluent cultures of (*a*) human sebocytes of the donor prior to transfection experiments (second subculture), (*b*) SZ95 cells (first subculture), and (*c*) SZ95/K7 cells (50th subculture) exhibit a similar epithelial, polymorphous appearance with cells of different sizes. *Scale bar*: 20 μm.

multiple donors, whereas prolonged experiments are hindered by the short life span of the cells.

To overcome this problem we transfected human facial sebocytes with Simian virus (SV)-40 large T antigen and immortalized them. The resulted cell line, termed SZ95, has been cloned and investigated further, and was shown to retain the characteristics of human sebocytes.

MATERIALS AND METHODS

Cell cultures Unless otherwise stated, all cells were maintained as adherent cultures in a standard medium constituted of modified DME medium/Ham's F 12 medium (1:1) (Biochrom, Berlin, Germany) with 2 mM *N*-acetyl-L-alanyl-L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (Biochrom) and 50 μg gentamicin per ml (Gibco-BRL, Karlsruhe, Germany) at 37°C in a humidified atmosphere containing 5% CO_2. Culture medium was replaced every 2–3 d.

Isolation and culture of human sebocytes Human sebaceous glands were isolated from the facial skin of a 87 y old female patient undergoing surgery, as previously described (Xia *et al*, 1989). The isolated glands were cultured without feeder layer in the standard medium supplemented with 9 ng epidermal growth factor per ml, 9 ng keratinocyte growth factor per ml (both Boehringer, Mannheim, Germany), 0.4 μg hydrocortisone per ml (Sigma, Munich, Germany), and 10^{-9} M cholera toxin (Calbiochem, Bad Soden, Germany). Primary human sebocyte cultures resulted as outgrowths from the periphery of the gland lobules.

Cultivation of human keratinocytes, fibroblasts, and HMEC-1 cells Human keratinocytes were isolated from the frontoparietal area of a 57 y old female patient undergoing surgery, were cultured by standard techniques (Boyce and Ham, 1983), and were maintained in serum-free keratinocyte growth medium which consisted of keratinocyte basal medium, 5 ng epidermal growth factor per ml, 50 μg bovine pituitary extract per ml (all Gibco-BRL), and 50 μg gentamicin per ml. The first and second subcultures were used in the studies. Human fibroblasts were used in passages between 1 and 3. The human microvascular endothelial cell line HMEC-1 (Ades *et al*, 1992), immortalized by the SV-40 large T antigen, was used in passages between 20 and 30.

Transfection of human sebocytes The vector used for the transfection of human sebocytes, designated as pSVT, is a PBR322-based construct containing the sequences encoding the transforming SV-40 large T protein and its expression is driven by the Rous sarcoma virus long-terminal repeat (Dutt *et al*, 1990; Wang *et al*, 1991). Human sebocyte cultures in the second subculture were grown to 50% confluency in 35 mm culture dishes (Becton Dickinson, Plymouth, U.K.) and were used for transfection performed by a standard gene transfer technique with lipofectin reagent (Gibco-BRL datasheet). Briefly, culture medium was removed, cell cultures were washed twice with Opti-MEM serum-free medium (Gibco-BRL), and incubated in Opti-MEM for 4 h. Opti-MEM was then replaced by an antibiotic-free transfection mixture which constituted of 1.5 ml Opti-MEM various lipofectin reagent (5–30 μl) (Gibco-BRL) and pSVT DNA concentrations (1–10 μg) in 0.5 ml phosphate-buffered saline. The cultures were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Finally, the cultures were washed twice with standard culture medium and further maintained in the sebocyte medium, as described above. Cells from each dish were observed for growth.

Detection of SV-40 large T antigen The expression of SV-40 large T antigen in transfected sebocytes, as well as in human keratinocytes, fibroblasts, and HMEC-1 cells was detected by immunocytochemistry and western blot analysis using a mouse serum antihuman SV-40 T antigen monoclonal antibody (MoAb) (Oncogene Science, Cambridge, MA) diluted 1:1000 for immunocytochemistry and 1:100 for immunoblotting.

Cloning of immortalized human sebocytes cultures (SZ95 cells) SZ95 cells of the tenth subculture were cloned by limited dilution in 96-well culture plates (Nunc, Wiesbaden, Germany) (one cell per well as detected by light microscopy). Single cells and the resulting clones were maintained in the standard medium supplemented with 5 ng epidermal growth factor per ml and 3 ng keratinocyte growth factor per ml.

Cytogenetic analysis For chromosome preparation mitotic SZ95 cells were harvested after 2 h of treatment with colcemid (Gibco-BRL). Following trypsinization, cells were treated with hypotonic KCl solution (75 mM) for 10 min and subsequently were fixed three times with ice-cold methanol/glacial acetic acid (3:1) (Merck, Darmstadt, Germany). GTG-banding followed a standard procedure (Wang and Fedoroff, 1972).

Characterization of SZ95 cells Representative cultures of SZ95 cells were characterized as follows and compared with human keratinocytes: (i) Cell morphology determined by inverted phase-contrast microscopy and electron microscopy as well as cell size measured by flow cytometry; (ii)

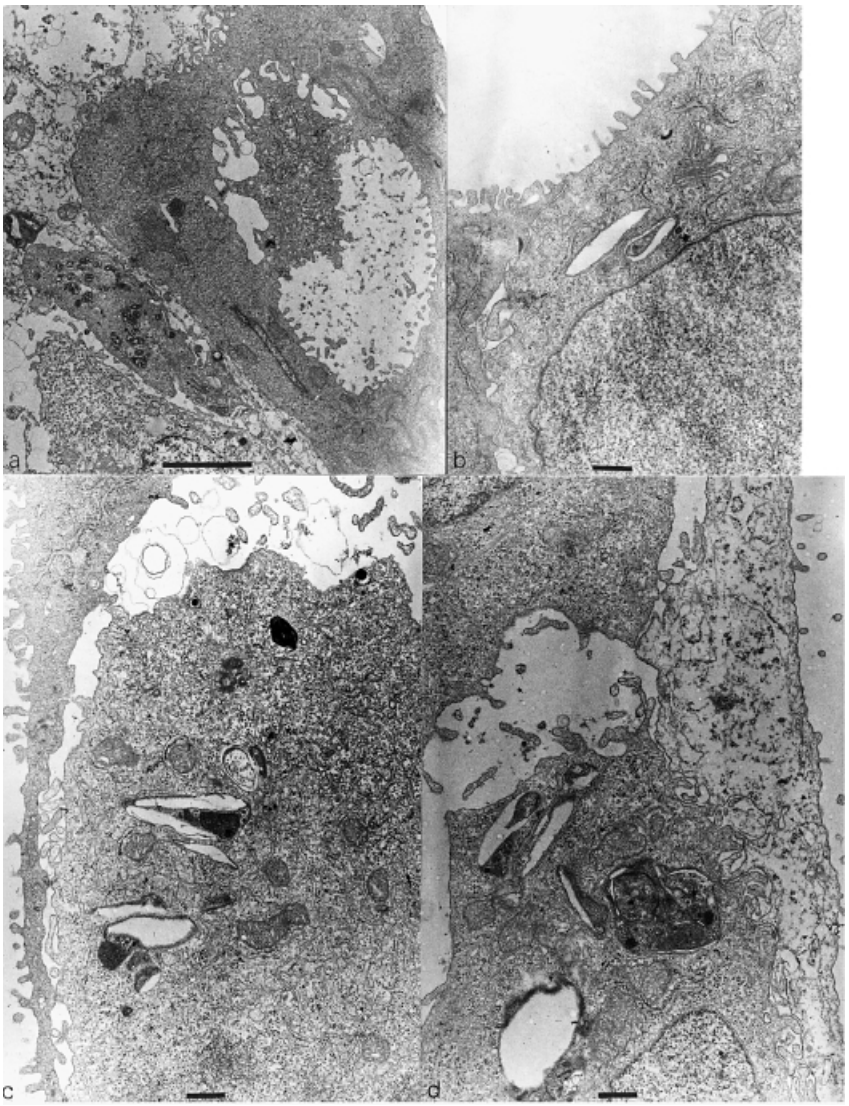

Figure 3. Electron microscopy. SZ95 cells show (*a*) microvillous transformation of their cell membranes and (*a, b*) large cytoplasm profiles with abundant organelles, especially Golgi membranes and ellipsoid vacuoles. Lack of or only few desmosomal areas are detected. (*c*) Several inclusion bodies, round and ellipsoid vacuoles as well as (*d*) myelin figures of various types are seen in the cytoplasm of SZ95 cells, indicating lipid synthesis. *Scale bar*: 0.47 μm.

expression of sebocyte markers (Zouboulis *et al*, 1991c, 1994) evaluated by immunocytochemistry and western blot analysis (**Table I**); (iii) cellular lipid distribution detected by Oil Red and Nile Red stains and flow cytometry of Nile Red stained cells as well as lipid synthesis assessed by high performance thin layer chromatography (HPTLC) of ^{14}C-acetic acid-labeled lipid fractions extracted from cultured cells and their supernatants; and (iv) pattern of cell proliferation and growth kinetics under the influence of 5α-DHT and retinoids.

Immunocytochemistry Dispersed cells of subconfluent cell cultures were attached to glass slides by cytocentrifugation in a Shandon Cytospin 2 device. Slides were air dried and fixed with cold acetone for 10 min. The cytocentrifuge preparations were subsequently incubated with the primary MoAb (**Table I**) and labeling was visualized using standard techniques (Zouboulis *et al*, 1994).

Isolation and quantitation of proteins Cell cultures were lysed directly in the culture dishes by a solution which consisted of 50 mM HEPES, 1% Nonidet p-40 (ICN, Aurora, OH), 150 mM NaCl, and a protease inhibitor (Complete Mini; Boehringer), were manually scrapped, harvested, and homogenized by ultrasonic disruption. All steps were performed at 4°C. After centrifugation of the cell homogenates, total protein of the supernatants was quantified by a standard bicinchoninic acid protein assay (Pierce, Rockford, IL) by measuring the absorbance at 550 nm in an Dynatech MR5000 enzyme-linked immunosorbent assay reader.

Western blot analysis One-dimensional sodium dodecyl sulfate/polyacrylamide gel electrophoresis of protein aliquots (20 μg) was performed on 7.5% gels. Proteins were transferred to an immobilon-P PVDF transfer membrane (Millipore; Eschborn, Germany) using a BioRad blotting system. The blots were subsequently incubated with the primary MoAb (**Table I**) and with 0.2 μg horseradish peroxidase per ml complex-conjugated goat anti-mouse MoAb or goat anti-rabbit MoAb (Oncogene Science) at steps of 60 min at room temperature. Secondary antibodies were detected by chemiluminescence (ECL; Amersham, Braunschweig, Germany) on Kodak XAR 5 X-ray films illuminated for several time points.

Electron microscopy For transmission electron microscopic examination cell cultures were grown to confluence on plastic Lux Thermanox coverslips (Miles Lab, Naperville, IL) and were processed using standard techniques (Detmar *et al*, 1989).

Oil Red and Nile Red staining Cells grown in chamber slides (Nunc) were incubated either with 0.6% Oil Red solution (Sigma) in 60% isopropanol for 15–120 min or with 1 μg Nile Red dye per ml (Kodak, Rochester, NY) for 15 min at room temperature, as previously described (Xia *et al*, 1989). The cultures were then observed under a light microscope (Oil Red stain) or a fluorescence microscope using a 450–500 nm bandpass exciter filter by light emission of > 528 nm (Nile Red stain).

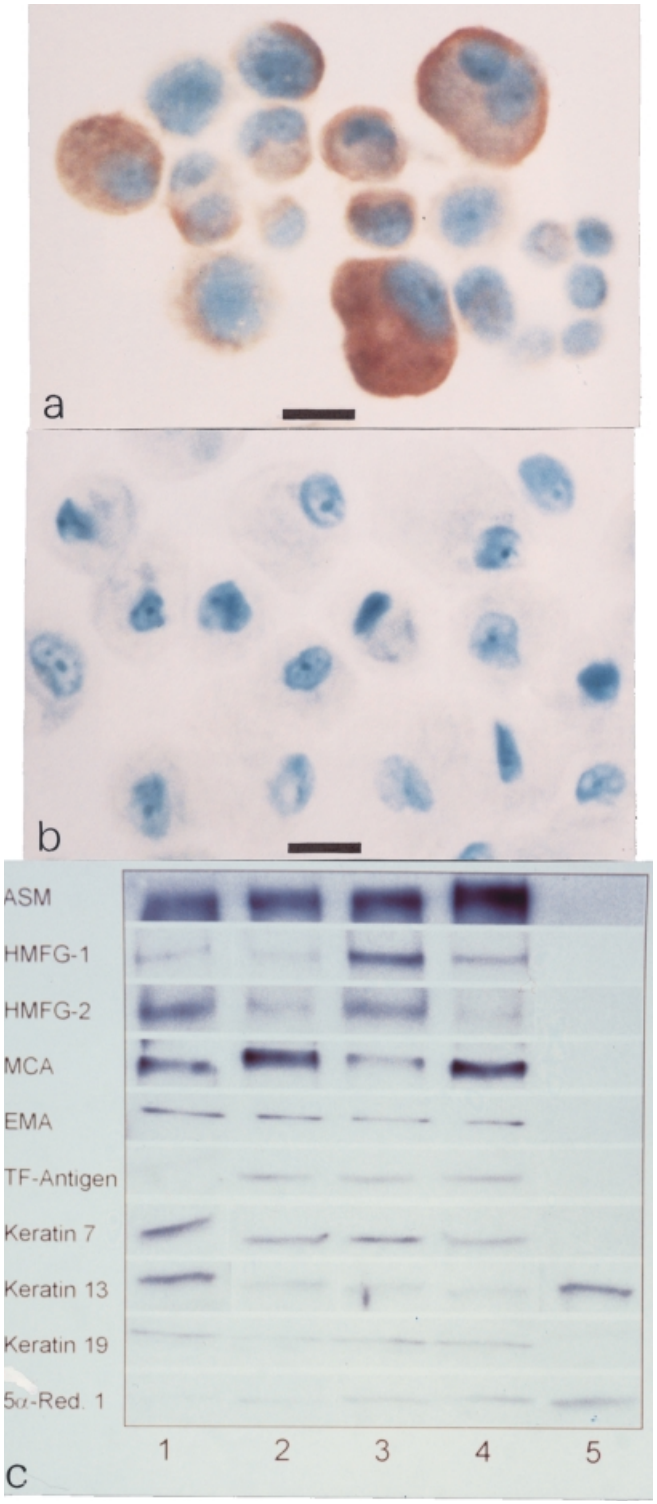

Figure 4. Detection of sebocyte proteins. Cytocentrifuge preparations of (*a*) SZ95 cells and (*b*) human keratinocytes labeled with the OM-1 MoAb against the sebaceous gland antigen. SZ95 cells, but not keratinocytes, shown positive cytoplasm staining. *Scale bar*: 3 μm. (*c*) Western blot analysis of SZ95 cells (34th subculture) (*lane 1*), SZ95/K6 cells (*lane 2*), SK95/K7 cells (*lane 3*), SZ95/K28 cells (*lane 4*), and human keratinocytes (*lane 5*) for expression of human epithelial sialo-mucin (ESM), human milk fat globulin-1 (HMFG-1), human milk fat globulin-2 (HMFG-2), mucin-like carcinoma-associated antigen (MCA), epithelial membrane antigen (EMA), Thomsen–Friedenreich antigen (TF-antigen), keratins 7, 13, and 19, and 5α-reductase type 1 (5α-Red 1). The SZ95 cell line and its clones expressed all investigated proteins, whereas keratinocytes only expressed keratin 13 and 5α-reductase type 1.

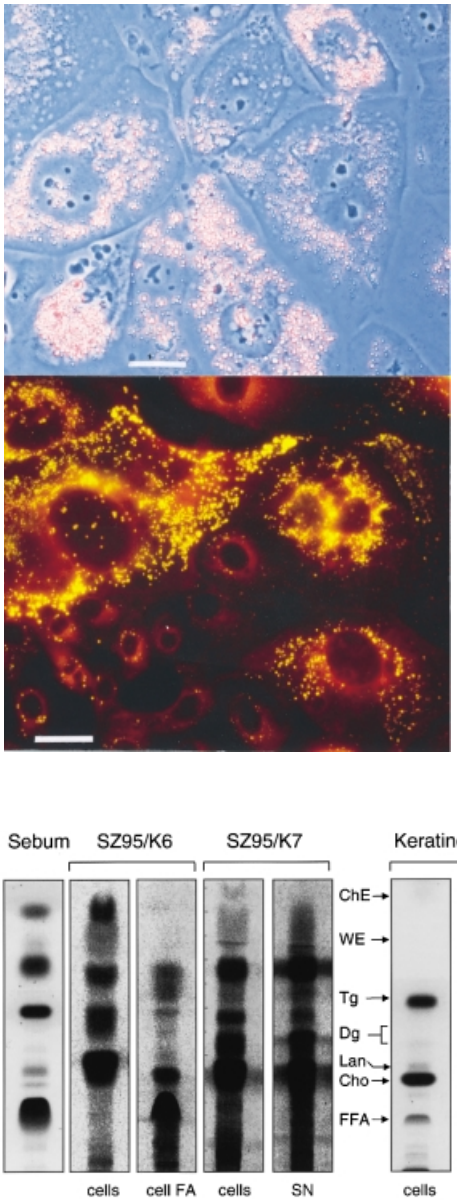

Figure 5. Synthesis of lipids. SZ95 cells positively labeled with (*a*) Oil Red dye and (*b*) Nile Red fluorescence dye identifying neutral lipids. *Scale bars*: 10 μm. (*c*) Storage imaging of HPTLC fractionated lipids synthesized in SZ95/K6 (*lanes 3* and *4*) and SZ95/K7 cells (*lanes 5* and *6*) been pulsed with 0.5 μCi [2-^{14}C]-acetic acid, sodium salt per ml (45–60 mCi per mmol) for 24 h. The cells (*lanes 3–5*) synthesize several fractions of neutral lipids, among them the sebaceous lipids squalene (Sq) and wax esters (WE) as well as triglycerides (Tg), cholesterol (Cho), cholesterol esters (ChE), diglycerides (Dg), lanosterol (Lan), and free fatty acids (FFA). Cholesterol and triglycerides were mainly isolated from human keratinocytes (*lane 7*), whereas wax esters were not identified. Neutral lipids were also found to a lesser extent in the supernatants (SN; *lanes 6* and *8*). *Lane 1*, lipid standards, *lane 2*, human sebum lipids, *lane 4*, fatty acids (FA) extracted from the SZ95/K6 cells

Flow cytometry Dispersed nonlabeled cells of confluent cultures were sorted for cell size, whereas confluent cells labeled with Nile Red were assessed for lipid content by standard flow fluorometric techniques. Ten thousand cells per sample were analyzed using a Panasonic fluorescence activated cell sorter.

Labeling and extraction of lipids Subconfluent cell cultures were maintained in standard medium for 2 d and then pulsed with 0.5 μCi [2-^{14}C]-acetic acid, sodium salt per ml (45–60 mCi per mmol; DuPont-NEN, Boston, MA) in RPMI-1640 medium supplemented with 10% heat-

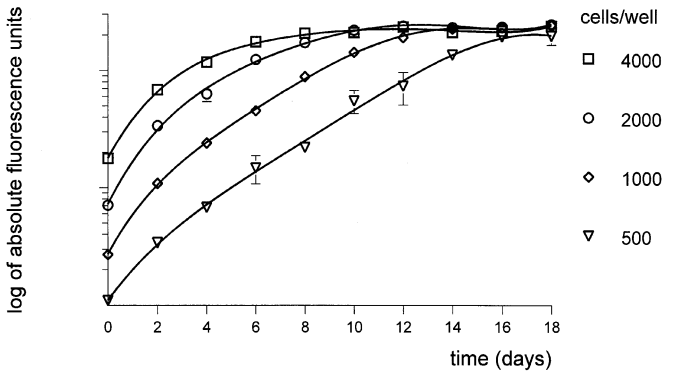

Figure 6. Cell kinetics. Proliferation of SZ95/K7 cells over 18 d. Seeding cell density influenced the duration of logarithmic proliferation. Logarithmic proliferation occurred from 2 to 14 d and population doubling times was 54.3 h at 500 cells per well, 2–10 d and 51.7 h at 1000 cells per well, 2.6 d and 51.3 h at 2000 cells per well, respectively. No logarithmic growth could be detected at a seeding density of 4000 cells per well. The log of the mean values ± SD of sextuplicate evaluations are presented.

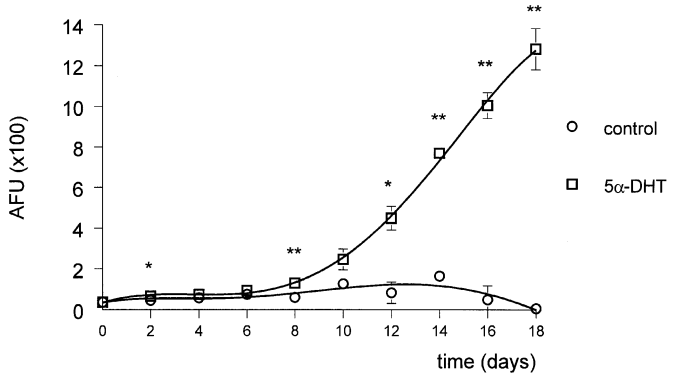

Figure 7. Effect of 5α-DHT on cell proliferation. Proliferation of SZ95/K7 cells (2000 cells per well) over 18 d in serum-free medium (control) and in serum-free medium supplemented with 10^{-6} M 5α-DHT. From day 8, 5α-DHT significant increased the proliferation of SZ95 cells, exhibiting population doubling times of 136 h (control) and 53.7 h (5α-DHT-treated cells). The log of the mean values ± SD of sextuplicate evaluations are presented. *$p < 0.05$; **$p < 0.01$

inactivated fetal bovine serum for an additional 24 h. Lipids were isolated from cells and their supernatants and separated into neutral lipids, fatty acids, and phospholipids, as previously described.[2] Briefly, the cells were lysed with distilled H_2O and the pellets were diluted in tetrahydrofuran/chloroform (1:2) (Merck) (Huang and Cabot, 1990). The culture media were mixed with methanol (Merck) (10% final concentration) and the solution was applied to reverse phase columns (LiChrolut C18; Merck) which have been prepared by subsequent washing with chloroform/methanol (2:1) and methanol. After a 10 min centrifugation at 10^3 r.p.m., the tetrahydrofuran/chloroform phase (cells) or methanol phase (supernatants) was collected and dried using a Life Sciences SpeedVac SC110A vacuum concentrator. For separation of the lipid mixtures into individual classes a modification of the two-phase separation method by Kaluzny *et al* (1985) was used utilizing aminopropyl bonded phase columns (Supelco-Sigma, Munich, Germany) to ensure complete recovery. Dry lipids were diluted in chloroform and passed through the N-hexane (Merck)-pretreated columns. Neutral lipids were eluted with chloroform/2-propanol (Merck) (2:1), fatty acids with 2% acetic acid in diethyl ether (both Merck), and phospholipids were finally separated by 0.1 M ammonium acetate in methanol (Merck). All lipid fractions were dried and diluted again in chloroform/methanol (2:1) immediately prior to HPTLC analysis.

HPTLC and assessment of lipid fractions Size-fractionation and visualization of neutral lipids and of free fatty acids was obtained by HPTLC carried out on 20 × 10 cm silica gel-coated glass plates (Merck). The plates were pretreated with N-hexane and left to dry for 24 h. The samples were applied on the plates by an automatic Camag Linomat IV applicator. Chromatograms of neutral lipids were developed to 9 cm in a N-hexane/diethyl ether solution (9:1), left to dry, redeveloped to 4.5 cm in a solution of chloroform/diethyl ether/ethyl acetic acid (80:4:16), and exposed to Fuji TR2040S storage imaging screens which were then scanned using a Fuji BAS 1000 bio-imaging analyzer. Prior to screen exposure, the lane with nonlabeled lipid standards (Sigma and Alexis, Läuselfingen, Switzerland) run together with the samples was cut, was heated on a Camag TLC Plate Heater III for 10 min to 180°C, was cooled, dipped in 10% $CuSO_4 \cdot 5H_20$ and 8.5% H_3PO_4 (both Merck) for 10 s, and then reheated for 30 min to 120°C to char the lipids. Phospholipids were counted by liquid scintigraphy. All data were related to the corresponding protein volumes.

Growth kinetics Cell cultures were seeded in 96 well culture plates at densities of 0.5–4 × 10^3 cells per well. Cell proliferation was assessed by the 4-methylumbelliferyl heptanoate fluorescence assay and measured automatically, as previously described (Zouboulis *et al*, 1991b). On the day of evaluation culture medium was removed, the cells were washed twice with phosphate-buffered saline and 100 µl of a 100 µg 4-methylumbelliferyl heptanoate per ml solution (Serva, Heidelberg, Germany) in phosphate-buffered saline were added to each well. The plates were then incubated at 37°C for 30 min and released fluorescence was read on a Flow Titertek Fluoroscan II. The results are given as absolute fluorescence units using 355 nm excitation and 460 nm emission filters.

Treatment with 5α-DHT 5α-DHT (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and subsequently in serum-free, phenol red-free standard medium supplemented with 50 µg bovine pituitary extract per ml, 1 mg fatty acid-free bovine serum albumin per ml (Boehringer), 5 ng epidermal growth factor per ml, and 50 µg gentamicin per ml giving final concentrations of 10^{-6} M 5α-DHT and 0.1% DMSO. DMSO (0.1%) alone served as the control. SZ95 cells (0.5–2 × 10^3 cells per well) were treated with 5α-DHT for 18 d.

Treatment with retinoids All-*trans*-retinoic acid, 13-*cis*-retinoic acid and acitretin were dissolved in DMSO and subsequently in serum-free standard medium supplemented with 50 µg bovine pituitary extract per ml, 1 mg fatty acid-free bovine serum albumin per ml, 5 ng epidermal growth factor per ml, and 50 µg gentamicin per ml giving final concentrations of 10^{-7} M retinoid and 0.1% DMSO. DMSO (0.1%) alone served as the control. Retinoids were handled under dim amber light. SZ95 cells and keratinocytes (0.5–1 × 10^3 cells per well) were treated with retinoids for 9 d.

Statistical analysis Growth studies were assessed in sextuplicate wells of 96 well plates. All other experiments were performed in triplicate dishes. Statistical significance of the data was evaluated by the two-sided Student's t test. Mean differences were considered to be significant when $p < 0.05$.

RESULTS

Transfection of human sebocytes with SV-40 large T antigen After the transfection procedure dramatically decreasing viability of pSVT-treated sebocytes was detected for 4 wk. This was followed by the emergence of a proliferating colony after use of 30 µl lipofectin reagent and 10 µg pSVT DNA. These cells, termed SZ95, have been passaged over 50 times to date and show no signs of senescence, being still viable after 4½ y.

Stability of SZ95 cell phenotype SZ95 cells have been investigated at passages 3 and 10. For both the uncloned cell line

[2]Seiffert K, Fritsch M, Ketteler R, Orfanos CE, Zouboulis CE: A novel method for separation of neutral lipids and phospholipids from cultured cells and their supernatants. *J Invest Dermatol* 108:375, 1997 (abstr.)

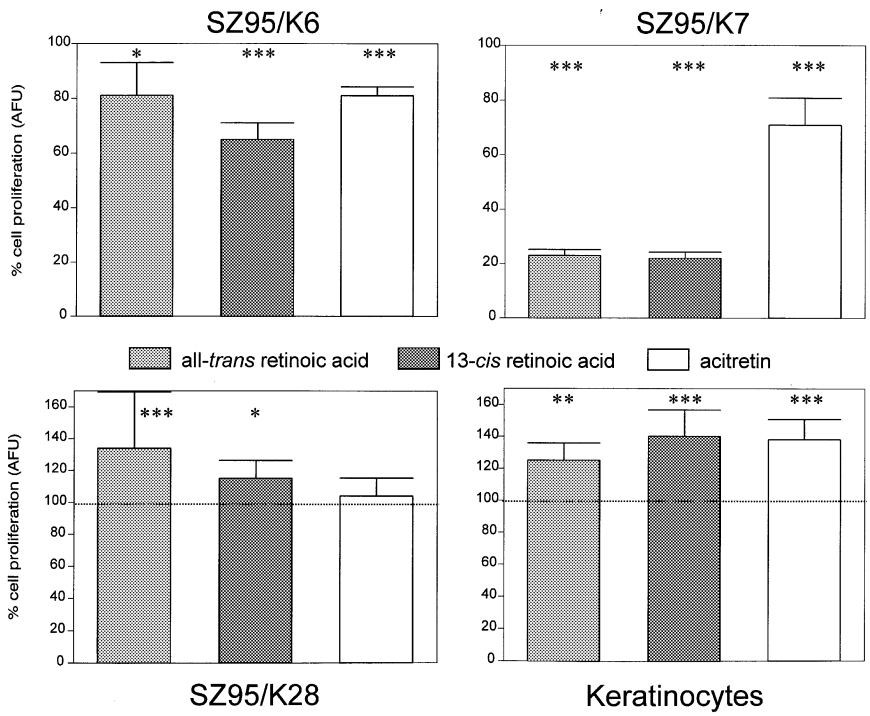

Figure 8. Effects of retinoids on cell proliferation. Addition of all-*trans*-retinoic acid, 13-*cis*-retinoic acid or acitretin (all 10^{-7} M) to SZ95/K6, SZ95/K7, SZ95/K28 cells and human keratinocytes maintained in serum-free medium (seeding density 1000 cells per well). SZ95/K6 and SZ95/K7 cell growth was significantly inhibited by retinoids in the magnitude 13-*cis*-retinoic acid > all-*trans*-retinoic acid > > acitretin, whereas SZ95/K28 cells and keratinocytes responded to retinoids with stimulation of cell proliferation. The mean values ± SD of sextuplicate evaluations at treatment day 9 are presented. Comparison has been made with untreated controls (100%). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

and the clones presented here studies have been repeated between passages 10 and 20, 20 and 30, 30 and 40, and 40 and 50. SZ95 cells presented an overall stable phenotype so that the experiments presented hereafter, between passages 34 and 50, are representative of the behavior of SZ95 cells.

Detection of SV-40 large T Antigen Immunocytochemical labeling of SZ95 cells and of HMEC-1 cells, used as a positive control, with MoAb against SV-40 large T protein revealed strong nuclear staining, whereas some cells also exhibited labeling of cytoplasm (**Fig 1**). Normal human keratinocytes and fibroblasts were uniformly negative for SV-40 large T protein. These data were confirmed by western blot analysis of SZ95 total proteins which detected a 94 kDa band, being consistent with the molecular weight of SV-40 large T protein, as previously reported (Harlow *et al*, 1981). As expected, nontransfected human sebocytes and keratinocytes did not express the 94 kDa band (**Fig 1*c***).

Cloning of SZ95 cells Several clones of SZ95 cells were obtained. Three clones, termed SZ95/K6, SZ95/K7, and SZ95/K28 have been further investigated. SV-40 large T protein expression in all three clones was confirmed by western blot analysis (**Fig 1*c***).

Cytogenetic analysis The SZ95 cell line and the clones SZ95/K6, SZ95/K7, and SZ95/K28 had highly hyperdiploid–aneuploid female karyotypes. They displayed polyploid populations due to frequently observed endoreduplications resulting in the formation of megakaryocytes. Furthermore, micronuclei were formed in all lines examined. The SZ95 cell line (44th subculture) exhibited chromosome numbers ranging from 60 to 67 (median 64.5 chromosomes, n = 10 cells); the modal chromosome number in the SZ95/K6 clone (40th subculture) was 54–62 (median 61 chromosomes, n = 10 cells), in the SZ95/K7 clone (40th subculture) 60–69 (median 63.5 chromosomes, n = 10 cells), and in the SZ95/K28 clone (40th subculture) 51–59 (median 55 chromosomes, n = 10 cells). The SZ95/K7 clone was examined again in the 47th subculture after adaptation in a phenol red-free sebocyte medium for seven passages and the modal chromosome number was 59–67 (median 64.5 chromosomes, n = 20 cells). In addition to the numerical chromosome aberrations, structural abnormalities were identified in all cell lines involving complex translocations between different chromosomes and the formation of dicentric, ring, and small marker chromosomes.

Phenotypic characterization of SZ95 cells The morphology of SZ95 cells was similar to those of nontransfected sebocytes, namely epithelial, exhibiting a polymorphous appearance with cells of different size, whereas numerous droplets could be seen in the cell cytoplasm (**Fig 2**). Cells of different sizes up to 3.2–3.25-fold in different experiments during proliferation and 5–6-fold at confluence were found by flow cytometry. Under the electron microscope, SZ95 cells showed microvillous transformation of their cell membranes and lack of or only few desmosomal areas. Cytoplasm profiles were large with abundant organelles, especially extended rough endoplasmic reticulum, Golgi apparatus, several inclusion bodies as well as round and elipsoid vacuoles and myelin figures of various types indicating lipid synthesis (**Fig 3**). None of these ultrastructure characteristics were found in human keratinocytes cultured in the same series of experiments.

SZ95 cells stained positively for the sebaceous gland antigen when examined by immunocytochemistry in contrast to keratinocytes which were not stained by the MoAb OM-1 recognizing the sebaceous gland antigen (**Fig 4**). Western blot analysis detected expression of keratins 7, 13, and 19 as well as of several proteins of the human polymorphous epithelial mucin family in SZ95 cells, whereas human keratinocytes only expressed keratin 13. As expected (Chen *et al*, 1998), both SZ95 cells and keratinocytes expressed 5α-reductase type 1.

Lipid synthesis Numerous cytoplasmic lipid droplets were detected in SZ95 cells by Oil Red and Nile Red staining (**Fig 5**) as well as by electron microscopy (**Fig 3**). SZ95 cells decreased their lipid content from 510 fluorescence units per cell (median value) when cultured with 10% fetal bovine serum to 429 fluorescence

units per cell (median value; −16%) in serum-free medium, as detected by flow cytometry of Nile Red-stained cells. The cells produced squalene and wax esters, as well as triglycerides and free fatty acids, even after 25–40 passages (**Fig 5**); cholesterol and triglycerides were mainly isolated from human keratinocytes, whereas wax esters have not been detected.

Proliferation studies A logarithmic proliferation pattern of SZ95 cells was determined under normal culture conditions with average population doubling times of 52.4 ± 1.6 h (**Fig 6**). Proliferation of SZ95 cells was reduced in serum-free medium (population doubling time of 136 h) and was retrieved after cell adaptation to serum-free conditions or addition of 5α-DHT (population doubling time of 53.7 h) (clone SZ95/K7) (**Fig 7**).

The different SZ95 cell clones investigated exhibited distinct behaviors to retinoids. Whereas the proliferation of SZ95/K6 and SZ95/K7 cells was significantly inhibited by retinoids in the expected magnitude 13-*cis*-retinoic acid > all-*trans*-retinoic acid > > acitretin, the proliferation of SZ95/K28 cells was stimulated by all-*trans*-retinoic acid and 13-*cis*-retinoic acid similar to the proliferation of human keratinocytes (**Fig 8**).

DISCUSSION

Cell immortalization has widespread utility in a number of different biologic systems, provided that the immortalized cells retain critical biologic features characteristic of the parent cells. Transfection of human cultured cells with SV-40 large T antigen seems to represent a successful formula. SV-40 large T antigen, the dominant oncogenic protein expressed by the SV-40 DNA virus, is able to form stable and functionally inactive complexes with host-cell proteins, such as p53, DNA polymerase and transcription factors AP-1 and AP-2, that act specifically as proliferation inhibitors (Mitchell *et al*, 1987; Dobbelstein *et al*, 1992) as well as to affect the mitotic regulatory proteins cyclin A, cyclin B, Cdc25C, and p34(cdc2) (Chang *et al*, 1997). Several immortalized cell lines have been obtained from normal human cells by transfection with SV-40 large T antigen, two of them being of epithelial origin, namely amniotic epithelial cells (Tohyama *et al*, 1997) and prostate epithelial cells (Bae *et al*, 1998).

In this study, human sebocytes derived from facial skin were stably transfected with SV-40 large T antigen as documented by the retained expression of SV-40 large T antigen even in late passages. We have used cationic lipids for sebocyte transfection, because this method has been found to be efficient for introducing DNA into human epidermal keratinocytes (Jiang *et al*, 1991; Staedel *et al*, 1994). Our cells were successfully immortalized, in that they have been passaged more than 50 times over the span of approximately 4½ y. In contrast, nontransfected human sebocytes can only be maintained for three to six subcultures with decreasing numbers of proliferating and increasing numbers of differentiated cells (Xia *et al*, 1989; Fujie *et al*, 1996). Like other cell lines immortalized with SV-40 large T antigen, SZ95 cells were found to be aneuploid cells with chromosomal abnormalities (Ades *et al*, 1992). Maintained in serum-free medium, SZ95 cells grew better than nontransfected human sebocytes (Zouboulis *et al*, 1993) and retained their capacity to synthesize sebaceous lipids in contrast to nontransfected cells. These data clearly demonstrate that SZ95 cells can serve as a continuously renewable cell line that can be grown in defined media.

SZ95 cells would be of limited utility if they failed to maintain a sebocyte-specific, stable phenotype. Indeed, SZ95 cells presented several characteristics of nontransfected human sebocytes, such as the presence of cells with different sizes (Zouboulis *et al*, 1994), a polymorphous epithelial appearance with numerous lipid droplets in their cytoplasm (Xia *et al*, 1989; Doran *et al*, 1991b; Zouboulis *et al*, 1991c), abundant cytoplasmic organelles as well as structures indicating lipid synthesis (Zouboulis *et al*, 1991c), and synthesis of the characteristic sebaceous lipids squalene and wax esters (Zouboulis *et al*, 1991a). On the other hand, synthesis of abundant amounts of free fatty acids in SZ95 cells even at late passages confirmed similar findings with nontransfected human sebocytes *in vitro* (Doran *et al*, 1991b; Zouboulis *et al*, 1991c; Fujie *et al*, 1996) invalidating previous concepts which suggested free fatty acids, found abundant in sebaceous follicles and acne lesions, to be a product of triglyceride metabolism induced by follicular bacteria.

To define further the phenotypic characteristics of SZ95 cells, expression of markers of sebocyte origin and differentiation *in vivo* and *in vitro* was examined. Polymorphous epithelial mucins are highly glycosylated high-molecular-weight glycoproteins expressed on the cell surface or released as secretory products by certain epithelial cells. In human skin and normal sebocytes *in vitro*, sebaceous gland antigen (Latham *et al*, 1989; Zouboulis *et al*, 1991c), milk fat globulin-2 (De Kretser, 1988), epithelial sialo-mucin (MAM-6) (Tsubura *et al*, 1987; Zouboulis *et al*, 1994), and epithelial membrane antigen (Xia *et al*, 1989) are mainly expressed in differentiating sebocytes. Milk fat globulin-1, MAM-3, Thomsen–Friedenreich antigen, and mucin-like associated antigen are expressed in human sebocytes but also in sebaceous gland duct cells and in sweat glands (Tsubura *et al*, 1987; de Kretser, 1988; Stähli *et al*, 1988; Kanitakis *et al*, 1998). SZ95 cells, like nontransfected human sebocytes, expressed all antigens of this family as well as keratins 7, and 19, and 13, and type 1 5α-reductase (Zouboulis *et al*, 1991c, 1994; Chen *et al*, 1998). The antigenic phenotype of SZ95 cells confirmed their sebocyte origin and sebaceous differentiation *in vitro* (Zouboulis, 1992) and clearly distinguished them from human keratinocytes.

SZ95 cells, especially clones K6 and K7, functionally behaved in a manner similar to nontransfected human sebocytes. They were found to respond to 5α-DHT by enhancing their proliferation *in vitro* (Akamatsu *et al*, 1992; Fujie *et al*, 1996). SZ95 cells were currently shown by our group to express and functionally use all enzymes required for intracellular androgen metabolism, including 3β-hydroxysteroid dehydrogenase/Δ^{5-4}-isomerase which converts dehydroepiandrosterone sulfate to androstenedione and is solely expressed in sebaceous glands in human skin (Milne, 1969; Dumont *et al*, 1992). They also express the androgen receptor.[3] Furthermore, a 5α-reductase type 1 inhibitor, but not 5α-reductase type 2 inhibitors, selectively inhibited testosterone reduction in SZ95 cells.[4]

In addition, SZ95/K6 and SZ95/K7 cells reduced proliferation under treatment with nonaromatic retinoids (13-*cis*-retinoic acid > *all-trans*-retinoic acid), whereas they barely responded to the aromatic retinoid etretinate (Zouboulis *et al*, 1991a; Doran *et al*, 1991a). On the contrary, SZ95/K28 cells behaved like human keratinocytes indicating that a part of the SZ95 cells probably lost the capacity of sebocyte-specific response to retinoids after transfection.

In conclusion, the immortalized human sebocytes of the SZ95 cell line were shown to retain their sebocyte identity by characterization and functional studies and could clearly be distinguished from human keratinocytes. SZ95 cells offer therefore unique possibilities for further investigations on the physiology of the sebaceous gland and its role in skin diseases. Moreover, this model also serves as an adequate tool for evaluating the biologic activity of different compounds on sebaceous gland cells.

We thank Dr. M. Fritsch and Dr. K. Seiffert of our Department for performing flow cytometry and lipid analysis. Following scientists and companies are acknowledged for kindly providing materials: Dr. E.W. Ades and Professor T.J. Lawley,

[3]Fritsch M, Orfanos CE, Zouboulis ChC: Androgen metabolism in the human sebocyte line SZ95. Submitted for publication.

[4]Seiffert K, Fritsch M, Seltmann H, Orfanos CE, Zouboulis ChC: Inhibition of 5α-reductase activity in immortalized sebocytes and keratinocytes *in vitro*. Submitted for publication.

*Emory University School of Medicine, Atlanta, GA (HMEC-1 cell line), Dr. A. Srinivasan, Thomas Jefferson University, Philadelphia, PA (pSVT construct), Dr. T.A. Kretser and M. Palatsides, Peter McCallum Cancer Institute, Melbourne, Australia (MoAb OM-1), Dr. Van Luu-The, CHUL Research Center and Laval University, Quebec, Canada (type 1 5α-reductase MoAb), Hoffmann-La Roche, Basel, Switzerland (all-*trans *retinoic acid, 13-*cis *retinoic acid and acitretin). Mrs M. Hoffmann and Mr A. Adic are acknowledged for skilled technical assistance. A part of these experiments constitutes the diploma work of H. Seltmann, former student of biology at the Free University of Berlin. H. Seltmann is a scholar of the Department of Dermatology, the Free University of Berlin. This work was supported by a research grant of the European Academy of Dermatology and Venereology and the Yamanouchi Europe B.V., as well as in part by grants of the Deutsche Forschungsgemeinschaft (Zo 75/2-1), of the Research Committee of the Medical Faculty, The Free University of Berlin, and of the MSD Sharp & Dohme GmbH, Germany.*

REFERENCES

Ades EW, Candal FJ, Swerlick RW, George VG, Summers S, Bosse DC, Lawley TJ: HMEC-1: Establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol* 99:683–690, 1992

Akai Y, Akamatsu H, Ri S, Ito A, Zouboulis ChC, Asada Y: Influence of free fatty acids on the proliferation of cultured human sebocytes in vitro. *Jpn J Dermatol* 104:647–649, 1994

Akamatsu H, Zouboulis ChC, Orfanos CE: Control of human sebocyte proliferation in vitro by testosterone and 5-alpha-dihydrotestosterone is dependent on the localization of the sebaceous glands. *J Invest Dermatol* 99:509–511, 1992

Bae VL, Jackson-Cook CK, Maygarden SJ, Plymate SR, Chen J, Ware JL: Metastatic sublines of a SV40 large T antigen immortalized human prostate epithelial cell line. *Prostate* 34:275–282, 1998

Baumrind NL, Parkinson D, Wayne DB, Heuser JE, Pearlman A: EMA: a developmentally regulated cell-surface glycoprotein of CNS neurons that is concentrated at the leading edge of growth cones. *Dev Dyn* 194:311–325, 1992

Boyce ST, Ham RG: Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J Invest Dermatol* 81(Suppl.):33s–40s, 1983

Brown SK, Shalita AR: Acne vulgaris. *Lancet* 351:1871–1876, 1998

Chang TH, Ray FA, Thompson DA, Schlegel R: Disregulation of mitotic checkpoints and regulatory proteins following acute expression of SV40 large T antigen in diploid human cells. *Oncogene* 14:2383–2393, 1997

Chen W, Zouboulis ChC, Fritsch M, *et al*: Evidence of heterogeneity and quantitative differences of the type 1 5α-reductase expression in cultured human skin cells. Evidence of its presence in melanocytes. *J Invest Dermatol* 110:84–89, 1998

Cooper MF, McGrath H, Shuster S: Sebaceous lipogenesis in human skin. Variability with age and severity of acne. *Br J Dermatol* 94:165–172, 1976

Cunliffe WJ: The sebaceous gland and acne—40 years on. *Dermatology* 196:9–15, 1998

De Kretser T: The ovarian-carcinoma-associated sebaceous gland antigen is a member of the HMFG2-positive family of epithelial mucins. *Tumor Biol* 9:154–164, 1988

Detmar M, Mayer-da-Silva A, Stadler R, Orfanos CE: Effects of azelaic acid on proliferation and ultrastructure of mouse keratinocytes in vitro. *J Invest Dermatol* 93:70–74, 1989

Dobbelstein M, Arthur AK, Dehde S, van Zee K, Dickmanns A, Fanning E: Intracistronic complementation reveals a new function of SV40 T antigen that co-operates with Rb and p53 binding to stimulate DNA synthesis in quiescent cells. *Oncogene* 7:837–847, 1992

Doran T, Shapiro SS: Retinoid effects on sebocyte proliferation. In: Packer L (ed.). *Methods in Enzymology*. San Diego: Academic Press, 1990, pp 334–338

Doran TI, Lucas DA, Levin AA, *et al*: Biochemical and retinoid receptor activities in human sebaceous cells. In: Saurat J-H (ed.). *Retinoids: 10 Years On*. Basel: Karger, 1991a, pp 243–253

Doran TI, Baff R, Jacobs P, Pacia E: Characterization of human sebaceous cells in vitro. *J Invest Dermatol* 96:341–348, 1991b

Dumont M, Luu-The V, Dupont E, Pelletier G, Labrie F: Characterization, expression, and immunohistochemical localization of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase in human skin. *J Invest Dermatol* 99:415–421, 1992

Dutt K, Scott M, Del Monte M, Agarwal N, Sternberg P, Srivastava SK, Srinivasan A: Establishment of human retinal pigment epithelial cell lines by oncogenes. *Oncogene* 5:195–200, 1990

Fujie T, Shikiji T, Uchida N, Urano Y, Nagae H, Arase S: Culture of cells derived from the human sebaceous gland under serum-free conditions without a biological feeder layer or specific matrices. *Arch Dermatol Res* 288:703–708, 1996

Geiger J-M: Retinoids and sebaceous gland activity. *Dermatology* 191:305–310, 1995

Gollnick H, Zouboulis ChC, Akamatsu H, Kurokawa I, Schulte A: Pathogenesis and pathogenesis related treatment of acne. *J Dermatol* 18:489–499, 1991

Harlow E, Crawford LV, Pim DC, Williamson NM: Monoclonal antibodies specific for simian virus 40 tumor antigens. *J Virol* 39:861–869, 1981

Hsia SL, Fulton JE, Fulgham D, Buck MM: Lipid synthesis from acetate-1–^{14}C by suction blister epidermis and other skin components. *Proc Soc Exp Biol Med* 135:285–291, 1970

Huang CF, Cabot MC: Phorbol diesters stimulate the accumulation of phosphatidate, phosphatidylethanol, and diacylglycerol in three cell types. Evidence for the indirect formation of phosphatidylcholine-derived diacylglycerol by a phospholipase D pathway and direct formation of diacylglycerol by a phospholipase C pathway. *J Biol Chem* 265:14858–14863, 1990

Jiang C-K, Konnolly D, Blumenberg M: Comparison of methods for transfection of human epidermal keratinocytes. *J Invest Dermatol* 97:969–973, 1991

Kaluzny MA, Duncan LA, Merritt MV, Epps DE: Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J Lipid Res* 26:135–140, 1985

Kanitakis J, Al-Rifai I, Faure M, Claudy A: Thomsen-Friedenreich and its precursor (Tn) antigen expression in normal skin and in benign cutaneous tumours: a marker for sebaceous differentiation. *Acta Derm Venereol (Stockh)* 78:173–176, 1998

Karsten U, Butschak G, Cao Y, Goletz S, Hanisch FG: A new monoclonal antibody (A78-G/A7) to the Thomsen-Friedenreich pan-tumor antigen. *Hybridoma* 14:37–44, 1995

Kealey T, Lee CM, Thody AJ, Coaker T: The isolation of human sebaceous glands and apocrine sweat glands by shearing. *Br J Dermatol* 114:181–188, 1986

Lane EB, Bártek J, Purkis PE, Leigh IM: Keratin antigens in differentiating skin. *Ann NY Acad Sci USA* 455:241–258, 1985

Latham JAE, Redfern CPF, Thody AJ, de Kretser TA: Immunohistochemical markers of human sebaceous gland differentiation. *J Histochem Cytochem* 37:729–734, 1989

Lee CM: Cell culture systems for the study of human skin and skin glands. In: Jones CJ (ed.) *Epithelia: Advances in Cell Physiology and Cell Culture*. Dordrecht: Kluwer, 1990, pp 333–350

Luu-The V, Sugimoto Y, Puy L, Labrie Y, Solache IL, Singh M, Labrie F: Characterization, expression, and immunohistochemical localization of 5α-reductase in human skin. *J Invest Dermatol* 102:221–226, 1994

Milne JA: The metabolism of androgens by sebaceous glands. *Br J Dermatol* 81:23–28, 1969

Mitchell PJ, Wang C, Tjian R: Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* 50:847–861, 1987

Nikkari T: Comparative chemistry of sebum. *J Invest Dermatol* 62:257–267, 1974

Ormerod MG, Steele K, Westwood JH, Mazzini MN: Epithelial membrane antigen: partial purification, assay and properties. *Br J Cancer* 48:533–541, 1983

Petersen MJ, Zone JJ, Krueger GG: Development of a nude mouse model to study human sebaceous gland physiology and pathophysiology. *J Clin Invest* 74:1358–1365, 1984

Pochi P: Sebaceous gland assay. In: Lowe N, Maibach H (eds). *Models in Dermatology*, Vol. 2. Basel: Karger, 1985, pp 70–75

Ramaekers F, Huysmans A, Schaart G, Moesker O, Vooijs P: Tissue distribution of keratin 7 as monitored by a monoclonal antibody. *Exp Cell Res* 170:235–249, 1987

Shapiro SS, Hurley J, Vane FM, Doran T: Evaluation of potential therapeutic entities for the treatment of acne. In: Reichert U, Shroot B (eds). *Pharmacology of Retinoids in the Skin, Pharmacol Skin*, Vol. 3. Basel: Karger, 1989, pp 104–112

Sharp F, Hay JB, Hodgins MB: Metabolism of androgens in vitro by human foetal skin. *J Endocrinol* 70:491–499, 1976

Staedel K, Remy J-S, Hua Z, Broker TR, Chow LT, Behr J-P: High-efficiency transfection of primary human keratinocytes with positively charged lipopolyamine:DANN complexes. *J Invest Dermatol* 102:768–772, 1994

Stähli C, Caravatti M, Aesbacher M, Kociba C, Takacs B, Carman H: A mucin-like carcinoma associated antigen (MCA) defined by three monoclonal antibodies against different epitopes. *Cancer Res* 48:6799–6802, 1988

Strauss JS: Sebaceous gland, acne and related disorders—an epiloque. *Dermatology* 196:182–184, 1998

Taylor-Papadimitriou J, Peterson JA, Arklie J, Burchell J, Ceriani RL, Bodmer WF: Monoclonal antibodies to epithelium specific components of the human milk fat globule membrane: production and reaction with cells in culture. *Int J Cancer* 28:17–21, 1981

Tohyama J, Tsunoda H, Sakuragawa N: Characterization of human amniotic epithelial cells transformed with origin-defective SV40 T-antigen gene. *Tohoku J Exp Med* 182:75–82, 1997

Tsubura A, Morii S, Ueda S, *et al*: Immunohistochemical demonstration of MAM-3 and MAM-6 antigens in normal human skin appendages and their tumors. *Arch Dermatol Res* 279:550–557, 1987

Van Muijen GNP, Ruiter DJ, Franke WW, Achtstätter T, Haasnoot WHB, Ponec M, Warnaar SO: Cell type heterogeneity of cytokeratin expression in complex epithelia and carcinomas as demonstrated by monoclonal antibodies specific for cytokeratins nos. 4 and 13. *Exp Cell Res* 162:97–113, 1986

Wang HC, Fedoroff S: Banding in human chromosomes treated with trypsin. *Nature New Biol* 235:52–54, 1972

Wang Y-C, Neckelmann N, Mayne A, Herskowitz A, Srinivasan A, Sell KW, Ahmed-Ansari A: Establishment of a human fetal cardiac myocyte cell line. *In Vitro Cell Dev Biol* 27A:63–74, 1991

Xia L, Zouboulis Ch, Detmar M, Mayer-da-Silva A, Stadler R, Orfanos CE: Isolation of human sebaceous glands and cultivation of sebaceous gland-derived cells as an in vitro model. *J Invest Dermatol* 93:315–321, 1989

Zouboulis ChC: Sebaceous cells in monolayer culture. *In Vitro Cell Dev Biol* 28A:699, 1992

Zouboulis ChC, Korge B, Akamatsu H, Xia L, Schiller S, Gollnick H, Orfanos CE:

Effects of 13-*cis*-retinoic acid, all-*trans*-retinoic acid and acitretin on the proliferation, lipid synthesis and keratin expression of cultured human sebocytes in vitro. *J Invest Dermatol* 96:792–797, 1991a

Zouboulis ChC, Garbe C, Krasagakis K, Krüger S, Schröder K, Orfanos CE: A fluorometric rapid microassay to identify anti-proliferative compounds for human melanoma cells in vitro. *Melanoma Res* 1:91–95, 1991b

Zouboulis ChC, Xia L, Detmar M, Bogdanoff B, Giannakopoulos G, Gollnick H, Orfanos CE: Culture of human sebocytes and markers of sebocytic differentiation in vitro. *Skin Pharmacol* 4:74–83, 1991c

Zouboulis ChC, Korge BP, Mischke D, Orfanos CE: Altered proliferation, synthetic activity, and differentiation of cultured human sebocytes in the absence of vitamin A and their modulation by synthetic retinoids. *J Invest Dermatol* 101:628–633, 1993

Zouboulis ChC, Krieter A, Gollnick H, Orfanos CE: Progressive differentiation of human sebocytes in vitro is characterized by increased cell size and altered antigenic expression and is regulated by culture duration and retinoids. *Exp Dermatol* 3:151–160, 1994

Zouboulis ChC, Xia L, Akamatsu H, *et al*: The human sebocyte culture model provides new insights into development and management of seborrhoea and acne. *Dermatology* 196:21–31, 1998