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Expression Profiling of Genes and Proteins in HaCaT Keratinocytes: Proliferating Versus Differentiated State

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Abstract The knowledge of the mechanism of keratinocyte differentiation in culture is still uncompleted. The emergence of new technologies, such as cDNA microarrays or 2D electrophoresis followed by mass spectrometry analysis, has allowed the identification of genes and proteins expressed in biological processes in keratinocytes. Here, we report a genome wide analysis of proliferating versus differentiated human HaCaT keratinocytes. We found that genes and proteins which take part in the cell cycle control, carbohydrate metabolism, cell auto-immunity, adhesion and cytokine signal transduction pathways were regulated in differentiated HaCaT keratinocytes. In addition, we identified seven proteins and 33 transcripts that had not been previously described as differentially expressed in proliferating versus differentiated HaCaT cells. Furthermore, some of these transcripts or proteins were similarly regulated in human primary keratinocytes and in human epidermis. The present study opens new areas of investigation in the comprehension of keratinocyte differentiation. J. Cell. Biochem. 93: 1048–1062, 2004.

Key words: human; keratinocytes; proteomics; gene expression; cell differentiation

The human epidermis is an epithelium undergoing a permanent turnover throughout life. This self-renewal process implies a certain level of keratinocytes proliferation, which is mostly restricted to the basal layer of the epidermis [Barrandon and Green, 1987; Fuchs and Segre, 2000; Lavker and Sun, 2000; Watt and Hogan, 2000]. When keratinocytes leave the basal layer, they migrate through the upper layers, where they undergo morphological and biochemical changes to achieve terminal differentiation. All these biochemical and morphological changes are the result of genes being turned on and off [Eckert et al., 1997]. The identification of transcripts and proteins differentially expressed is being currently studied using large scale technologies. In this context, the effects of UV [Li et al., 2001; Sesto et al., 2002], those of TNFα and TGF-β have been studied on primary cultures of keratinocytes or on HaCaT cells using SAGE or microarrays analysis [Jansen et al., 2001; Zavadil et al., 2001]. These studies led to the identification of signaling and metabolic pathways activated by these treatments. On the other hand, large scale study led to establish a specific signature of

Abbreviations used: DTT, dithiothreitol; FCS, fetal calf serum; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; RT-PCR, reverse transcription-polymerase chain reaction; SAGE, serial analysis of gene expression; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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keratinocytes versus that of dermal fibroblast and melanocytes in human skin [Curto et al., 2002]. Another study focused on the analysis of both the transcriptome and the proteome of stem cell enriched keratinocytes compared to those of Transient Amplifying enriched keratinocytes [O'Shaughnessy et al., 2000] has allowed the identification of PA-FABP as a marker of Transient Amplifying keratinocytes. Finally, van Ruissen et al. [2002] described a partial transcriptome of the epidermis by SAGE analysis using a model where keratinocyte differentiation was induced by growth factor depletion before cell density reached confluence. While few studies have been performed using macro or microarrays, extensive studies have succeeded in establishing a protein catalogue using 2D-electrophoresis. Indeed, Celis and collaborators identified more than 100 proteins regulated during the differentiation of keratinocytes in primary cultures [Celis et al., 1995; Olsen et al., 1995].

The aim of the present study was to identify transcripts and proteins allowing a partial characterization of proliferating versus differentiated human keratinocytes using global approaches. For this purpose, we used the HaCaT cell line which is the most widely studied keratinocyte cell line. This cell line is capable of proliferating, differentiating [Boukamp et al., 1988], forming an epidermal organization in organotypic culture in vitro [Schoop et al., 1999; Maas-Szabowski et al., 2003] and, in vivo, of forming an epidermis when transplanted on nude mice [Breitkreutz et al., 1998]. In post-confluent HaCaT cell line, proliferative capacity is abolished and cells exhibit a phenotype of differentiated keratinocytes expressing genes which are localized specifically in the upper layers of healthy skin [Ryle et al., 1989; Kato et al., 1995; Garach-Jehoshua et al., 1998]. In addition, ultrastructural studies demonstrated that confluent HaCaT cells exhibit differentiated structures seen as desmosomes [Capone et al., 2000]. Among the methods described to differentiate HaCaT cells in vitro, we choose to use cell density combined with high calcium concentration. In the present study, the comparison of the molecular phenotype of proliferating HaCaT keratinocytes to that of differentiated keratinocytes was assessed by comparing cells at 3 days and at 10 days of culture after plating. Results obtained from the HaCaT cells study were confirmed for some genes and proteins in human keratinocyte primary cultures and in normal human skin. The present work contributes to the description of genes and proteins whose expression profiles characterize human differentiated keratinocytes.

MATERIALS AND METHODS

Cell Culture and Cell Cycle Analysis

The keratinocyte cell line HaCaT was kindly provided by Dr. N. Fusenig [Boukamp et al., 1988]. HaCaT keratinocytes were seeded at 10,000 cells/cm^2 and cultured in DMEM containing 1.8 mM calcium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. Cells were either cultured for 3 days (*d3*), during the exponential growth phase, or for 10 days (*d10*), which correspond to 3 days after confluence is reached. In the later condition, keratinocytes differentiate rapidly due to high calcium concentration, high cell density, and nearly complete growth arrest. For cell cycle analysis, HaCaT cells were rapidly rinsed with PBS and trypsin followed by trypsin dissociation treatment (10 min at 37°C). Cells were then fixed, stained with propidium iodine, and filtered on a 10 μm nylon filter [Tani et al., 2000]. Flow cytometry was performed on a MoFlo analyzer (Cytomation). To eliminate doublets, we used a two dimensional dot-plot of peak fluorescence versus integrated signal. Single cells fall on the diagonal whereas doublets appeared above diagonal.

Human keratinocytes were isolated from foreskin biopsies and primary cultures were initiated on a feeder layer of lethally irradiated 3T3-J2 cells using DMEM and Ham's F12 media (3:1 mixture) containing 1.8 mM calcium, 10% FCS and supplemented as described by Dellambra et al. [2000]. Secondary cultures were seeded at 10,000 cells/cm^2 as described for HaCaT cells and collected three days later, when cells were highly dividing, or at ten days after plating.

Protein Purification and 2-D Gel Electrophoresis

Cells were resuspended in lysis buffer (Tris-HCl 40 mM pH 7.4, CHAPS 2%, urea 8M), and then treated with DNase I and centrifuged at 10,000*g* for 10 min at 4°C. Sub-cellular fractionation was performed as described [Siomi et al., 1996].

For the analysis of protein patterns, 100 μg of total proteins were first separated along linear immobilized pH-gradient strips (4–7) using

IPGphor apparatus (Amersham Biosciences, Buckinghamshire, UK). Proteins were separated using standard 15% SDS–PAGE and stained with silver nitrate (Amersham Biosciences). The same protocol was used for mass spectrometry (MS) analysis, except that the quantity of proteins loaded onto the strips was different (700 μg of proteins recovered from the various sub-cellular fractions) and gels were stained with Coomassie Blue (Bio-Rad, Hercules, CA). The molecular weight of proteins was determined using broad range molecular weight standards (Bio-Rad).

Protein Digestion and MALDI-TOF Analysis

Spots of interest were excised from 2-D gels, washed, and digested as described [Garin et al., 2001]. Mass spectra of the tryptic digests were acquired on a Biflex apparatus (Bruker-Franzen Analytik) and the peptide mass fingerprint was analyzed by MS-FIT (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm) or ProFound (http://129.85.19.192/prowl-cgi/ProFound.exe) softwares.

Antibodies and Western Blot Analysis

Anti-CEA (CD66e), HLA-DR, involucrin, PCNA, and cyclin B antibodies were purchased from Novacastra (Newcastle, UK). Anti-GAPDH antibody was purchased from Biodesign (Saco, ME). Anti-ER72 antibody was a generous gift from Dr. E. Chevet (McGill University, Montreal, PQ, Canada).

Total proteins (50 μg) extracted from 10^6 cells were loaded on 10 or 12.5% SDS–PAGE. Proteins were electro-transferred onto a PVDF membrane (Millipore, Bedford, MA). The Western blots were probed using anti-HLA-DR or anti-cyclin-B specific antibodies diluted at 1:100; anti-CD66e specific antibodies diluted at 1:200; anti-involucrin, anti-GAPDH, anti-PCNA, or anti-ER72 specific antibodies diluted at 1:500. Once washed, membranes were incubated with HRP-coupled species-specific antibody and cross reactions were revealed using a ECL chemiluminence kit (Amersham Biosciences).

Immunohistochemistry

Samples of normal skin and sections were prepared as described [Sivan et al., 2002]. The primary antibody was revealed by the LSAB®2 kit (DAKO) or directly by a Fluorescein (FITC)-conjugated Anti-Rabbit or Mouse IgG secondary antibodies (Jackson ImmunoResearch, West Grove, PA). For DAB staining, slides were submitted to 10 min incubations with biotin-linked antibody and peroxidase-labeled streptavidin followed by a diaminobenzidine treatment and a final counterstaining using haematoxylin.

Generation and Post-Processing of cDNA Microarrays

Gene expression analysis was performed using cDNA microarrays containing 2,496 human PCR products selected by direct interrogation of the Unigene database (http://www.ncbi.nlm.nih.gov/UniGene/). Primer pairs for the amplification of these ORFs were obtained using Primer 0.5 software (http://www.genome.wi.mit.edu/ftp/distribution/software/primer.0.5/) and the specificity of each primer sequence was verified by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). Primer pairs were designed to produce a 400–600 bp PCR product each ORF. PCR amplification of each gene was performed using a mix of cDNA obtained from various cell lines as a template. The PCR products were prepared in 96-well plates, purified by ethanol precipitation, washed in 70% ethanol, dried, dissolved in TE/DMSO (50/50), and stored at −80°C. The quality, size, and concentration of each PCR product were assessed by agarose gel electrophoresis using a calibrated standard DNA solution (Invitrogen, Carlsbad, CA). PCR products were arrayed onto poly-L-lysine-coated slides (Menzel Glaser, Braunschweig, Germany) using the 4-pins GMS 417 arrayer (Genetic Microsystems, Woburn, MA). A complete description of the microarrays has been deposited into the GEO Database [Edgar et al., 2002] at NCBI (www.ncbi.nlm.nih.gov/geo/) and is freely available under accession numbers GPL259 and GPL263.

Before hybridization, slides were hydrated and spotted DNA was cross-linked by UV irradiation (130 mJ at 254 nm). Slides were immersed in a freshly-prepared blocking solution consisting of 2% succinic anhydride, 20 mM sodium borate pH 8 in methyl-2-pyrrolidinone, then washed in water and dried in 100% ethanol at −20°C before centrifugation for 7 min at 100*g* at room temperature. Slides were then prehybridized at 42°C in 3× SSC, 0.1% SDS, 0.1% BSA solution for 30 min, washed and dried.

RNA Isolation, cDNA Synthesis and Hybridization

Total RNA was isolated using the Trizol reagent (Invitrogen) and dissolved in RNAse-free water. RNA quality was checked by 1% agarose gel electrophoresis and concentration was measured by absorbance at 260 nm.

Target cDNA was generated by direct incorporation using fluorescent nucleotide analogues Cy3 and Cy5 during reverse transcription of 20 μg of total RNA. Description of the protocols of labeling, hybridization and washing are available at NCBI under GEO accession number GSM4674.

Image Acquisition and Data Analysis

Fluorescence intensities of Cy3 and Cy5 were measured separately at 532 and 635 nm with a laser scanner (Axon Genepix4000B). The resulting 16-bit data files were imported into an image analysis software (Axon Genepix 2.0, Axon). Feature ratios were calculated after background subtraction using Genepix 2.0. This software flags spots as absent based on spot characteristics. Other bad spots were manually flagged and no flagged spots were included in subsequent analysis. The data file for each hybridization are available under the GEO accession number GSE336. Data were scaled so that the average median ratio values for all spots were normalized to 1.0. Additionally, only spots with more than 70% of their pixels greater than two standard deviations above background noise in one of the two wavelengths were selected for further analysis. An arbitrary cut-off ratio of twofold was used to select genes as significantly up or down-regulated relative to the control sample.

RT-PCR Analysis

Four micrograms of total RNA, extracted from primary keratinocyte cultures, were submitted to reverse transcription using oligo d(T) primers and Superscript II (Invitrogen) enzyme and controls were performed in presence of RNA without reverse transcriptase. PCR reactions were performed using either control reverse transcription experiments or primary keratinocyte cDNA as template. Specific primers corresponding to genes newly identified in keratinocytes were chosen in different exons to avoid amplification of genomic DNA.

RESULTS

Characterization of Proliferating and Differentiated HaCaT Keratinocytes

The HaCaT cells were cultured for 3 or 10 days and the cell cycle analysis was assessed by flow cytometry. At *d3*, 55% of cells were in S and G2/M phases, whereas at *d10*, 85% of cells were in G0/G1 phase (data not shown). This demonstrates that, under our experimental conditions, cells were either in a proliferative phase (*d3*) or in a confluent growth-arrested phase (*d10*) corresponding to a model of differentiated keratinocytes.

To confirm that HaCaT cells differentiate under our experimental conditions, we showed by RT-PCR that keratin 10 and involucrin are overexpressed at day 10 (results not shown).

Identification of Proteins Expressed in HaCaT Keratinocytes by Mass Spectrometry

The proteins extracted from HaCaT cells at *d3* or at *d10* were recovered after a partial subcellular fractionation followed by a 2D gel electrophoresis separation (data not shown). This partial purification allowed us to analyze several proteins, including some less abundant ones. The Coomassie blue staining of 2D gels performed with *d3* and *d10* protein extracts revealed the apparent presence or the apparent absence of 15 spots, which were excised from the gels and analyzed by mass spectrometry.

The apparent expression levels of four proteins isolated from the cytoplasmic fraction (Table I) increased at *d10* when compared to *d3*: Hsp 27, involucrin, cathepsin D precursor, and stratifin (14-3-3 sigma). On the other hand, the expression levels of 11 proteins decreased at *d10*: stathmin, CCT β, EF1A, cyclophilin A, DJ-1, triosephosphate isomerase (TPI), TPT-HRF, cytokeratin 8, PTB-associated splicing factor, ER 60, and cytoskeletal tropomyosin.

The number of proteins isolated from the subfractionation steps was greater than that obtained from total protein extracts. Some of these proteins were recovered in the expected sub-cellular fractions: TPI, TPT-HRF, and cyclophilin A were found to be located in the cytoplasmic fraction and PTB in the nuclear fraction. Others, which are known to be cytoplasmic proteins (cytokeratin 8, tropomyosin, ER-60), were found to be associated with the nuclear fraction. This result was not surprising since these proteins are part of the cytoskeleton and endoplasmic reticulum, thus being easily co-purified

TABLE I. Proteins Differentially Expressed in HaCaT Keratinocytes

Protein name	SWISSPROT accession number	MW[a] kDa	pI[a]	Recovery fraction	Danish-biosun database[b]	*d10* vs *d3* RNA ratio by microarray	Comments
Proteins in differentiated HaCaT							
Involucrin	P07476	125	4.5	Cytosol	+	3.0	Keratinocyte insoluble envelope
HSP-27	P04792	26	5.3	Cytosol	+	2.4	Stress resistance and actin organization
Cathepsin D	P07339	32	6.0	Cytosol	§	5.4	Lysosomal protease
Stratifin	P31947	30	4.4	Cytosol	+	3.2	P53 regulated inhibitor of G2/M progression; 14-3-3 family
Proteins in proliferating HaCaT							
CCT-beta	P78371	61.5	6.3	Membrane	+	0.3	Folding of tubulin
Stathmin	P16949	16	5.8	Membrane	–	0.4	Tubulin phosphorylation
Cyclophilin A	P05092	18	6.8	Cytosol	–	*	Cis-trans isomerase folding protein
ER-60	P30101	56.5	5.7	Nuclear	–	1.0	Protein disulfide isomerase
TPI	P00938	25.5	>7	Cytosol	+	0.3	Triosephosphate isomerase; glycolysis
TPT-HRF	P13693	24	4.6	Membrane	+	9.7	Acute allergic response
DJ-1	JC5394	25	6.8	Cytosol	–	0.7	Regulator of the androgen receptor
PTB	P23246	40	>7	Nuclear	–	*	Pre-mRNA splicing factor
Tropomyosin	P09493	36.5	4.4	Nuclear	+	0.9	Actin-associated protein
Cytokeratin 8	P05787	51	5.6	Nuclear	–	1.2	Cytoskeleton; intermediate filament
EF1A	P04720	57	4.2	Membrane	–	*	Binding of aminoacyl-tRNAs to 80S ribosomes

2D electrophoretic separation of proteins recovered at *d3* and *d10* followed by mass spectrometry analysis allowed the identification of differentiated and proliferating HaCaT expressed proteins.

[a]MW and pI were experimentally determined (see Materials and Methods).

[b]The results of the present study were compared to those described in the Danish 2D-keratinocyte database: (+) results in agreement with the database; (–) not determined in the Danish database; (§) not in agreement with the database; (*) the corresponding genes are not present in cDNA collection spotted on microarray.

with the nuclear fraction. Finally, some proteins were identified in the membrane fraction (Table I) even though none of them are known to be located in membranes. This finding was not surprising since membrane proteins cannot be easily separated by 2-D electrophoresis.

Identification of Genes Expressed in HaCaT Keratinocytes by cDNA Microarrays

Total RNA was extracted from HaCaT cells and cDNA was labeled with Cy3 or Cy5 dyes during reverse transcription. We performed ten independent hybridization experiments using a dye swap procedure. Six experiments were performed using *d3* HaCaT RNA labeled with Cy3 and four others using *d3* RNA labeled with Cy5. Four independent HaCaT cultures were used for RNA extraction. Of the 2,496 genes spotted on each array, an average of 1,370 genes was analyzed following the criteria described in Materials and Methods. A gene was considered to be significantly up or down-regulated with an arbitrary cut-off ratio of twofold and if the absolute ratio's value was greater than 2 in at least five experiments. Following the cross analysis of ten hybridization experiments, 89 genes were considered to be up-regulated and 82 genes down-regulated in differentiated HaCaT cells (see Table II).

We compared the relative levels of numerous transcripts expressed in the HaCaT cell line at *d3* to that of HaCaT at *d10*. Some genes corresponding to known markers of the differentiation of keratinocytes like involucrin, keratin 10, or Hsp 27 were found to be up-regulated at *d10*. Whereas, some genes like keratin 5 or keratin 14 [Poumay and Pittelkow, 1995] were not found to be significantly overexpressed at *d3* (results not shown), some other genes which are known to be preferentially expressed in the basal layer of the epidermis like cell cycle genes were clearly confirmed in this study (Table II).

Western Blot Analysis of Proteins Differentially Expressed in HaCaT Cells

In order to confirm some of the data obtained from the present analysis of protein and/or gene expression, we performed western-blotting on HaCaT cells (Fig. 1). Western blotting experiments with antibodies against four proteins expressed in proliferative HaCaT (cyclin B (Fig. 1A), PCNA (Fig. 1B), GAPDH (Fig. 1C), and ER72 (Fig. 1D), a protein disulphide isomerase very similar to ER60), revealed that their expression was diminished in differentiated HaCaT cells.

In addition, antibodies raised against three up-regulated proteins: involucrin (Fig. 1G), which is a keratinocyte differentiation marker; CEA, an adhesion molecules coded by PSG genes (Fig. 1F); and HLA-DR histocompatibility molecules (Fig. 1E), confirmed the data obtained from microarray and 2D electrophoresis analyses.

Gene and Protein Expression in Human Primary Keratinocytes and Human Skin

An important result of this study concerns the identification of 33 genes, never before described in the keratinocyte differentiation process, which were found to be regulated under our experimental conditions in the HaCaT cell line (see Table II). The expression and the regulation of these genes were then validated using primary cultures of human normal keratinocytes. Cells were cultured either in exponential growth phase or at confluence using the same cell-density experimental protocol used to induce HaCaT differentiation. Using classical RT-PCR experiments, we confirmed the expression of all these genes in human normal keratinocytes, except for UCP2 (Fig. 2A, lane 6) which exhibits unspecific multiple bands and ZNF211 (Fig. 2A, lane 7) which was not amplified in normal primary keratinocytes.

In addition, in order to confirm our microarray analysis, the 16 and 17 genes, newly identified which were found to be respectively up-regulated and down-regulated in differentiated HaCaT cells were analyzed by RT-PCR experiments on proliferating versus differentiated normal primary keratinocytes. Under these conditions, we found that 11 genes were upregulated and 11 genes were downregulated in differentiated human primary keratinocytes in culture (see Fig. 2B). For example, stathmin was found to be down regulated in differentiated normal keratinocytes (Fig. 2B), confirming results obtained from our protein data and microarrays analyses in HaCaT (Tables I and II).

Finally, we confirmed by RT-PCR (Fig. 3A) and immunohistochemistry results concerning four specific genes on HaCaT and normal keratinocytes. Stratifin and cathepsin D were shown to be preferentially expressed in the upper layers of normal epidermis (Fig. 3B)

TABLE II. Up and Down-Regulated Genes in Differentiated HaCaT Cells as Revealed by Microarray Study

Gene name	Fold change	GenBank accession number	Comments
Cell immunity			
HLA-A[a]	3.5	X02457	Histocompatibility antigen class I
HLA-DPB1[a]	2.9	M28202	Histocompatibility antigen class II
HLA-DQB1	3.1	M60028	Histocompatibility antigen class II
HLA-DRB1	4.2	X03069	Histocompatibility antigen class II
HLA-DRA	6.2	M60334	Histocompatibility antigen class II
B2M[a]	2.8	AB021288	Beta 2-microglobulin
CLU	4.8	M25915	Complement protein
C3	2.5	K02765	Complement component
RAB6A[b] (1)	2.1	M28212	MHC trafficking
UGB[a]	5.6	U01101	CC10, Anti-inflammatory modulation
TPT1[a]	9.7	X16064	Histamine releasing factor, acute allergic response, HLA class II synthesis
Cell adhesion and cytoskeletal			
PSG4[b]	10.2	M94891	Adhesion (CEA family)
PSG5[b]	3.2	M73713	Adhesion (CEA family)
PSG7[b]	6.9	U18467	Adhesion (CEA family)
PSG9[b] (16)	12.2	M34481	Adhesion (CEA family)
CEACAM1[b]	12.4	M72238	Biliary glycoprotein, cell surface recognition
LAMB3	3.3	D37766	Laminin-5 beta 3 chain, adhesion
BPAG1	2.7	M69225	Hemidesmosome
ROCK1[b] (2)	2.1	U43195	Rho-associated kinase
KRT 10	9.9	M19156	Keratin 10
KRT 13	3.5	X14640	Keratin 13
KRT 15	4.0	X07696	Keratin 15
SPTAN1	2.5	J03528	Actin cross-linking, spectrin
INV	3.0	M13903	Involucrin, keratinocyte envelope
LGALS3	2.6	AB006780	Laminin-binding protein
TUBB2[a]	−2.4	X03541	Beta-2 tubulin, microtubule cytoskeleton
TUBB5[a]	−2.5	X00734	Beta-5 tubulin, microtubule cytoskeleton
STMN1[b] (25)	−2.8	X53305	Stathmin, tubulin phosphorylation
FN1	−4.1	M10905	Fibronectin
KRT18[a]	−2.4	M26326	Keratin 18
EFEMP1[a]	−2.9	U03877	S1-5, fibrillin-like molecule
ICAP-1A[b] (26)	−2.5	AF012023	β1 integrin-associated protein
CLTB[a]	−2.3	M20470	Clathrin light-chain B, coated pit structure
CFL1[a]	−2.0	D00682	Cofilin, intracellular actin-modulating protein
ACTB[a]	−2.6	X00351	Beta actin
CD99	−2.1	M16279	Cell adhesion
Protease and inhibitors			
CTSB	5.4	M11233	Cathepsin D, lysosomal protease
MMP10[a]	2.3	X07820	Collagenase
SLPI[b] (3)	4.5	AF114471	Anti-leucoprotease
CAPNS1	2.6	X04106	Calcium dependent protease
Metabolism enzymes			
GFPT1[b] (4)	2.8	M90516	Hexosamine biosynthetic pathway
PYGB[a]	4.2	U47025	Glycogen phosphorylase
SAT[a]	4.0	M77693	Polyamine metabolism pathway
PSAP	4.3	J03077	Sphingolipid hydrolase
ODGH[b] (5)	2.5	D10523	2-oxoglutarate dehydrogenase
UCP2[b] (6)	3.1	U82819	Mitochondrial energy-consuming process
HADHB	2.2	D16481	Fatty acid beta-oxidation
BCKDHB	2.7	U50708	Valine, leucine, and isoleucine degradation
PCCA	3.0	X14608	Propanoate metabolism
ASS	5.7	X01630	Urea cycle
PGAM1[a]	−2.5	J04173	Phosphoglycerate mutase
PGK1[a]	−2.7	V00572	Phosphoglycerate kinase
TPI1[a]	−2.5	M10036	Triosephosphate isomerase
GAPD[a]	−2.5	M17851	Glyceraldehyde-3-phosphate dehydrogenase
ENO1	−2.2	M14328	Alpha enolase
FH[a]	−2.3	U59309	Fumarase hydratase (TCA)
FABP5	−2.2	M94856	Fatty acid binding protein
NP	−2.2	X00737	Purine metabolism
ODC1	−3.0	M16650	Ornithine decarboxylase
Transcription, cell cycle			
ZNF211[b] (7)	2.1	U38904	Zinc finger protein
ID1	3.5	X77956	bHLH-inhibitor of DNA binding
PBX1	2.6	M86546	homeo box transcription factor
HBP1[a]	3.6	AF019214	HMG box containing protein 1
RUNX1[b] (8)	2.5	D43969	AML-1 transcription factor
ADAR	3.2	U10439	Adenosine desaminase-RNA editing
PRP8[b] (9)	5.3	AB007510	Splicing factor
GRN[a]	3.3	M75161	Granulin, growth regulation

(*Continued*)

TABLE II. (*Continued*)

Gene name	Fold change	GenBank accession number	Comments
TP53	2.3	K03199	Cellular tumor antigen
CDKN1A	2.5	L25610	Waf1, cyclin dependent kinase inhibitor
BTG1[b] (10)	3.9	X61123	p53-dependent antiproliferative protein
PTGES[b] (11)	2.8	AF010316	Pig-12, p53-induced quinone oxydoreductase
TK1[a]	−2.5	K02581	Thymidine kinase, S phase
DTYMK[a]	−2.6	L16991	Thymidylate kinase (cdc8), S phase
TYMS[a]	−3.6	X02308	Thymidylate synthase, S phase
DHFR[a]	−3.4	J00140	Dihydrofolate reductase, S phase
CCNA2[a]	−4.2	X51688	Cyclin A, S phase
CCNB1[a]	−5.0	M25753	Cyclin B, G2/M checkpoint
CCND3	−2.3	M92287	Cyclin D3, G1-S phase
CDC2	−5.3	D88357	Cdc2 delta T, G2/M checkpoint
CKS2	−2.5	X54942	G2/M checkpoint
GSPT1[a]	−2.1	X17644	G1-to-S phase transition 1
PCNA	−2.9	M15796	G1–S phase
RFC4[a]	−2.9	M87339	Replication factor C subunit, PCNA associated
H4FG[a]	−3.2	X60486	H4 histone, nucleosome structure
RAD51[a]	−3.2	D14134	Repair of double-strand breaks
E2F1	−3.0	U47677	Transcription factor
MYBL2[a]	−3.4	X13293	B-myb, transcription factor
BRCA1[a]	−2.7	U14680	Transcription factor
ILF2[b] (17)	−2.0	U10323	NF45, transcription factor
SSBP1[b] (18)	−2.0	M94556	Single stranded DNA binding protein
HMG1[b] (19)	−2.3	D63874	Binds linear DNA
SSRP1[b] (20)	−2.0	M86737	High mobility group box, HMG1 homolog
TRIP13[a]	−3.3	U96131	HPV16E1 binding protein
SNRPE	−2.7	M37716	Ribonucleoprotein
RANBP1[b] (21)	−2.5	D38076	Formation of the mitotic spindle
HPRT[a]	−2.7	M31642	DNA repair and/or control of cell cycle progression
POLR2L[a]	−2.2	U37690	RNA polymerase II subunit, hsRPB10
G22P1	−2.0	S38729	Ku autoantigen p70, DNA repair
XRCC5	−2.3	J04977	Ku autoantigen, DNA repair
ADPRT[b] (22)	−2.5	M18112	Poly(ADP-ribose) polymerase, DNA repair
TEP1[b] (23)	−2.3	U86136	Telomerase-associated protein
CBX5	−3.5	L07515	Heterochromatin component
Cytokines and signal transduction			
TNFSF10	8.0	U37518	TRAIL, TNF-related apoptosis inducing ligand
VEGF[a]	2.7	AF022375	Angiogenic growth factor
TGFβ2	3.2	M19154	Growth factor
BMP1[a]	3.4	M22488	Growth factor
IL15	3.6	U14407	Cytokine
CASP1	2.0	M87507	IL1 convertase
MET[a]	2.9	J02958	HGF receptor
IGF2R	2.8	J03528	Growth factor receptor
EGFR	3.9	U48722	Growth factor receptor
ERBB2	2.7	M11730	Her-2, EGF receptor
IFNAR1	2.5	J03171	Interferon receptor
ACVRL1[a]	3.3	L17075	Activin receptor, TGFβ superfamily receptor type I
SCYA2[a]	5.8	X14768	MCP-1, chemokine involved in immunity
MCP2[a]	6.4	Y16645	Chemokine-MCP-1 family
STAT3	3.0	L29277	IL and IFN transduction pathway
JAK1	3.3	M64174	IL and IFN transduction pathway
GBP1[a]	2.9	M55542	IFN inducible protein
TAX1BP1[b] (12)	2.2	U33821	Tax1-binding protein-TNF pathway
GRF2[b] (13)	4.0	D21239	Crk or Grb2/Ash-associated protein
PLA2G2A	9.9	M22430	Phospholipids metabolism pathway
SFN	3.2	X57348	Stratifin, 14-3-3 family
THBS1[a]	−8.3	X14787	Thrombospondin 1, angiogenic factor
BMP7	−2.3	M60316	Bone morphogenic protein, growth factor
AR	−3.0	AH002608	Amphiregulin, EGF-related protein
CDC42	−2.4	M57298	Rac, GTP-binding protein
SERPINB2	−3.6	M18082	PAI2, Plasminogen activator inhibitor
SERPINB5	−2.8	U04313	Maspin, serine proteinase inhibitor
YWHAQ[b] (30)	−2.0	X56468	14.3.3 protein theta, signal transduction
SEMA3A	−2.4	L26081	Growth cones guidance
Chaperones			
HSPB1	2.1	L39370	Hsp 27, chaperone
ORP150[a]	2.5	U65785	Oxygen-regulated protein, ER molecular chaperon
CCT6A[a]	−2.5	M94083	Biogenesis of actin structures
HSPE1[a]	−3.3	U07550	Chaperonin 10, Hsp60-associated chaperone
HSPD1	−2.4	M34664	Hsp60, mitochondrial chaperonin
CCT2[a]	−2.5	AF026293	Folding of beta tubulin
DNAJA1[b] (24)	−2.4	L08069	Heat-shock protein, folding
Oxydative stress			
GPX2	3.1	X68314	Glutathione peroxidase

(*Continued*)

TABLE II. (*Continued*)

Gene name	Fold change	GenBank accession number	Comments
PRDX-1[a]	−2.5	L19184	Peroxiredoxin 1
SOD-1[a]	−2.7	K00065	Superoxide dismutase
GSTTLp28	−2.3	U90313	Glutathione-S-transferase
TXN	−2.1	J04026	Thioredoxin
Others			
BIRC3[a]	2.5	U37546	IAP homolog C-apoptose
CARS[a]	2.3	L06845	Cysteinyl-tRNA synthetase
AARS[a]	2.5	D32050	Alanyl-tRNA synthetase
TM7SF1[a]	4.0	AF027826	Putative 7 transmembrane protein
TFRC	3.2	M11507	CD71, transferrin receptor
GNS[a]	2.5	Z12173	Glucosamine-6-sulfatase, heparanase
STAG2[b] (15)	2.8	Z75331	SA-2, nuclear protein
MVP[a]	2.8	X79882	Drug resistance
MRP[a]	4.9	L05628	Drug resistance
NEDD4[b] (14)	4.3	D42055	KIAA0093, Unknown
HFL1	3.6	M65293	Lipid metabolism
EIF4A2	2.2	D30655	Protein synthesis initiation factor
S100A8	9.4	X06234	Calcium binding protein
PSMB9	2.8	U01025	Proteasome beta 9 subunit
DJ742c19.2	−6.5	U03891	Phorbolin I, specific keratinocyte protein
PDCD5[b] (27)	−3.0	AF014955	TFAR19, programmed cell death
UMPS[a]	−2.2	J03626	UMP synthase, pyrimidine biogenesis
GMPS[a]	−2.6	U10860	Guanosine 5′-monophosphate synthase
C2F[a]	−3.1	U72514	Unknown function
KPNA2[b] (28)	−3.7	U09559	Rch1, nuclear transport
RRM2[a]	−5.4	X59618	Small subunit ribonucleotide reductase
EIF4G1[b] (29)	−2.8	AF104913	Protein synthesis initiation factor
HMMR[a]	−2.8	AF032862	Intracellular hyaluronic acid binding protein
PSMB2[a]	−2.0	D26599	Proteasome subunit HsC7-I
PSMD3[a]	−2.8	D67025	Proteasome subunit p58
VDAC3[b] (31)	−2.4	AF038962	Voltage dependent anion channel, calcium channel
PPIH[b] (32)	−2.3	AF016371	USA-CyP, UsnRNP-associated cyclophilin
FRDA[b] (33)	−2.4	U43747	Frataxin, mitochondrial iron sequestration

Genes are grouped according to their functions. More detailed function of each gene is noted in the last column. GenBank accession numbers are indicated. Fold change corresponds to the ratio of fluorescence as described in "Materials and Methods". The values are the mean of ratios obtained in five or more microarray experiments.
[a]These genes were not previously described during keratinocyte differentiation process.
[b]Genes newly identified in keratinocytes. Parentheses numbers depicted the lane number of RT-PCR analysis described in Figure 2A.

whereas GAPDH was preferentially expressed in its basal layer. Finally stathmin was expressed in the basal and first suprabasal layers of the epidermis (Fig. 3B). Altogether, these results confirmed those obtained in vitro at the RNA and protein levels.

DISCUSSION

In the present study, we used proteomic methods and microarray technologies in order to set up a molecular profile characterizing HaCaT keratinocyte differentiation and validated some of our findings in cultured human normal keratinocytes and in human epidermis. Gene expression analysis allowed us to confirm previous data concerning genes known to be involved in differentiation and to identify genes not previously described in this process. Although the methods used in the present study were not exhaustive and the number of transcripts represented on the arrays remained limited to about 10% of the estimated number of genes in the human genome [Lander et al., 2001], we were able to identify 171 genes regulated in proliferating and differentiated HaCaT keratinocytes (for details see Table III). In addition, 33 of these 171 genes had never before been shown to be expressed in human keratinocytes. The comparison of gene expression analysis to that of protein expression revealed that for overexpressed proteins, all corresponding genes were up-regulated in differentiated keratinocytes (Table I). Likewise, four transcripts, which were repressed in differentiated keratinocytes, had their corresponding translation product clearly down-regulated. However, for three repressed proteins, the ratios of transcripts between *d10* and *d3* were unchanged and the transcription pattern of one gene (HRF) was the opposite of that of the protein (Table I).

Results obtained in this study demonstrated that transcripts and proteins found to

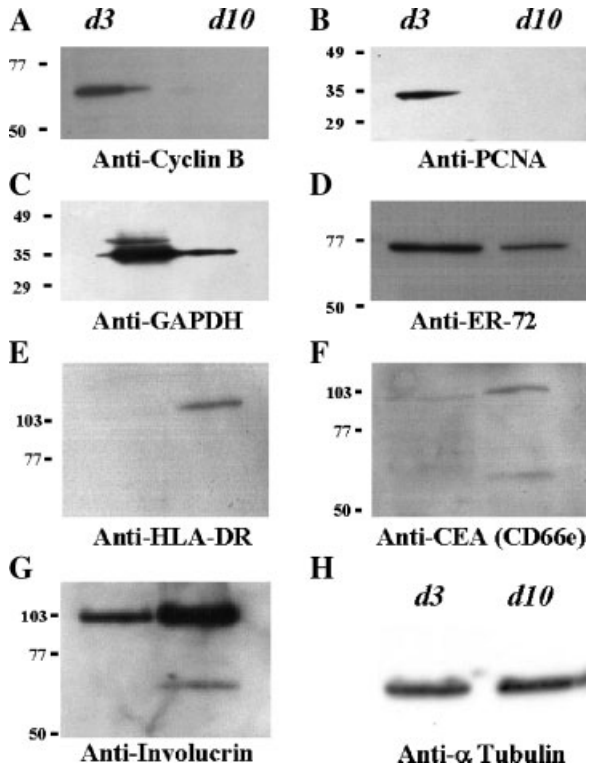

Fig. 1. Immunoblots of differentially expressed proteins in HaCaT cells. Total protein (50 μg) extracted from 10^6 HaCaT cells at *d3* and *d10* was loaded on SDS–PAGE, electro-transferred onto a nylon membrane, and proteins were specifically revealed using antibodies. **A**–**D**: Expression of proteins over-expressed at *d3*; (**E**–**G**) expression of proteins over-expressed at *d10*. Alpha tubulin (**H**) was shown as a representative loading control.

be modulated in differentiated HaCaT may be classified into five different biological functions such as cell cycle, cell adhesion, immunity, metabolism, and signal transduction. The role of these genes and their respective proteins in each of these biological functions are discussed below.

Cell Cycle

Microarray data analysis clearly showed that genes involved in different steps of the cell cycle are regulated during this process, especially in the S phase, cell cycle checkpoint, and mitosis. This was confirmed by cell cycle flow cytometry analysis, which showed that HaCaT cell growth was arrested in G0/G1 phase at *d10*.

In differentiated HaCaT cells, some genes involved in DNA replication were repressed, for example, SSBP1 and RCF4, which have been shown to play a key role in the initiation of replication [Niki et al., 2000]. In addition, PCNA has been found to be preferentially expressed in the basal layer of the normal epidermis. This gene, which plays an important role in the S phase [Miyagawa et al., 1989; Schmidt et al., 2001], was shown here to be down-regulated at the transcriptional level in differentiated HaCaT cells.

Furthermore, we observed a down-regulation of genes involved in the cell cycle control, especially those of the cyclin family: cyclin B, CKS2 as well as CDC2. These genes are known to interact and to participate in the formation and regulation of the maturation promoting factor during the G2/M checkpoint of the cell cycle [Egan and Solomon, 1998; Patra et al., 1999]. For example, the results concerning cyclin B were confirmed at the protein level by immunoblot analysis (see Fig. 1A). Other genes coding for proteins involved in cell division and proliferation like RANBP1 [Ren et al., 1995], tubulin [Karsenti and Vernos, 2001], stathmin and CCT2 [Yokota et al., 1999], are down-regulated in differentiated HaCaT cells. Finally, genes of the p53 family and associated proteins like Waf-1 (Table II), which were up-regulated at *d10*, are known to control cell cycle arrest.

Altogether, these results demonstrate that during HaCaT differentiation the expression of a significant number of genes implicated in cell division was strongly down-regulated at the transcriptional level. In addition, protein synthesis seemed to be activated, in differentiated HaCaT cells, since some genes coding for tRNA synthetases were up-regulated at *d10* (Table II).

Cell Adhesion and Immunity

During proliferation, the HaCaT cells exhibited low levels of mRNA encoding adhesion molecules whereas, in differentiated cells, several genes belonging to this family were shown to be up-regulated. The most significant transcriptional up-regulation was observed for CEA family molecules encoded by PSG genes. These results were confirmed by Western blot experiments for one member of the CEA family of proteins (CD66e) (see Fig. 1F). These proteins, co-expressed with differentiation markers like involucrin during the hair cycle [Honda et al., 1997], can be considered as markers of differentiation, since they have been described to be down-regulated in hyper proliferative tissues [Wilkinson et al., 2001]. In addition, it is interesting to note that six members of the CEA–CAM cluster located on chromosome

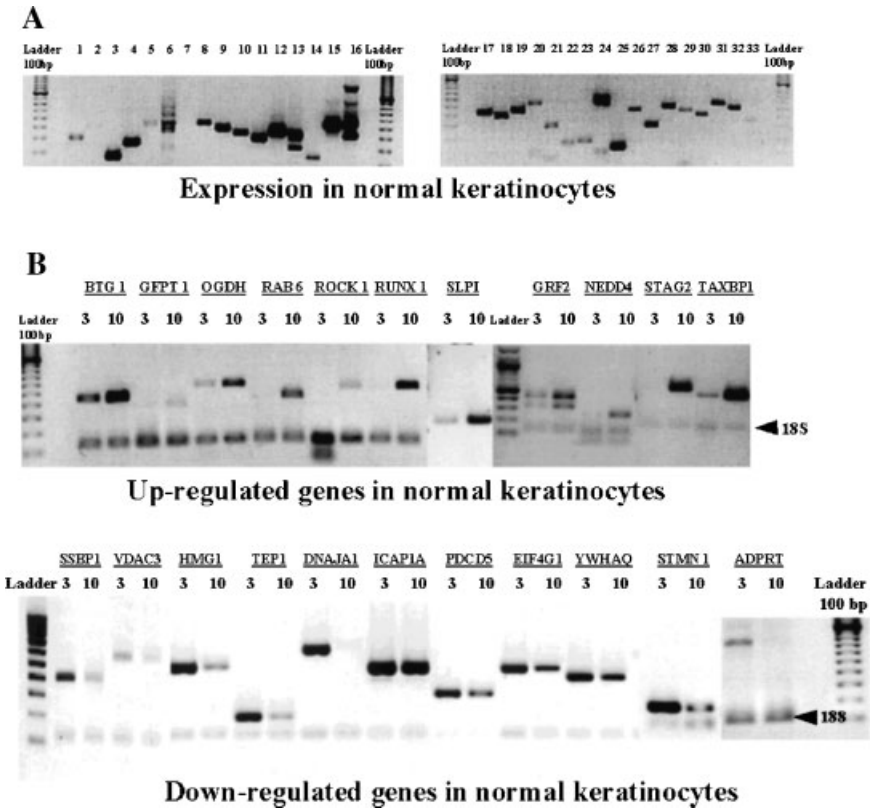

Fig. 2. Expression in human normal keratinocytes of genes newly identified in HaCaT cells. **A**: Total RNA from human normal keratinocyte was submitted to reverse transcription and PCR was performed using specific primers corresponding to genes found to be regulated in HaCaT cells. The number assigned to each line corresponds to genes listed in Table II. **B**: PCR performed on human normal keratinocytes cDNA at day 3 and day 10 of some differentially expressed genes using 18S probe in duplex as control.

19q13.2-13.3 [Brandriff et al., 1992] were shown to be up-regulated, indicating a possible common mechanism of trans-activation [Thompson et al., 1990].

Another significant change in differentiated HaCaT cells was observed for genes participating in the cell's self-immunity. A high number of class I and class II histocompatibility antigens were up-regulated under our culture conditions. Other proteins, like complement proteins or proteins involved in MHC trafficking (rab6) or degradation (cathepsins), were shown to be over-expressed in our study, suggesting that HLA antigen synthesis and degradation pathways were activated in HaCaT keratinocytes at *d10*. We also observed an up-regulation at *d10* of genes coding for secreted immunity-related chemokines named MCP [Robinson et al., 1989; Alam et al., 1994] as well as for HRF, a gene involved in histamine release [Kang et al., 2001]. Therefore, our results emphasize the role of the epidermis as an epithelial barrier involved in immune processes.

Metabolic Pathways

In differentiated HaCaT cells, microarray analysis showed that 5 of the 10 enzymes implicated in the glycolysis pathway were transcriptionally down regulated. For two of these genes (TPI and GAPDH), the down-regulation was confirmed at the protein level (see Table I and Figs. 1C and 3). These results corroborate previous studies showing a decreased uptake of glucose in the suprabasal layers of epidermis [Freindek and Traczyk, 1976]. In addition, beta-enolase is located in the basal layer of epidermis [Zieske et al., 1992], thus suggesting that this metabolic pathway is preferentially used by proliferative keratinocytes. The role of this pathway in terminal keratinocyte differentiation remains to be determined.

Growth Factors, Cytokine, and Signaling Pathways

Further analysis of microarray data indicated that a large number of genes coding for

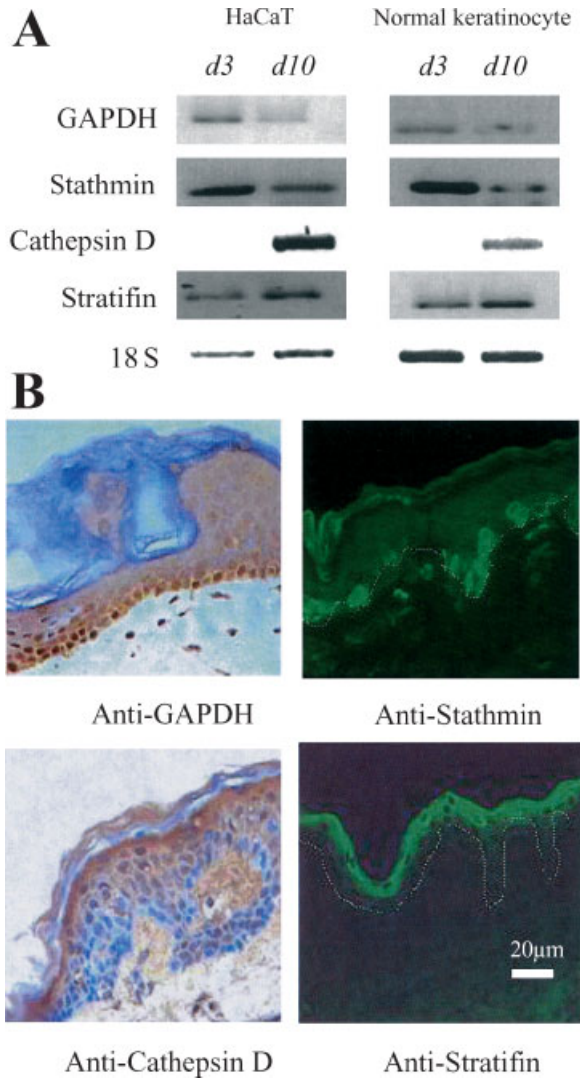

Fig. 3. Immunolocalisation of GAPDH, stathmin, cathepsin D, and stratifin in healthy human skin. **A**: RT-PCR analysis of selected genes on HaCaT and primary normal keratinocyte revealed the same pattern of expression between the two keratinocyte models. **B**: Proteins encoded by down-regulated genes (GAPDH and stathmin)and protein encoded by up-regulated genes (cathepsin D and stratifin) were localized in healthy human epidermis by immuno-histochemistry. DAB labeling was used for GAPDH and cathepsin D. Immunofluorescence coupled antibodies were used for stathmin and stratifin. For immunofluorescence, basement membrane is represented by a dotted line.

cytokines or growth factors and their receptors were up-regulated in differentiated HaCaT cells. For example, mRNA coding for the interferon (IFN) alpha receptor 1 was found to be up-regulated as well as three mRNAs (STAT3, JAK1, GBP1) coding for proteins involved in the IFN signal transduction pathway [Kotenko and Pestka, 2000]. These results suggest that differentiated HaCaT cells may exhibit a similar phenotype to that of activated keratinocytes which is observed during the wound healing process. It is noticeable that other genes coding for growth factors and their receptors involved in the same type of process were also up-regulated. Differentiated HaCaT cells expressed genes coding for stratifin, TRAIL, and TAX1 binding protein, which have been shown to be involved in terminal keratinocyte differentiation and cell death [De Valck et al., 1999; Dellambra et al., 2000; Qin et al., 2001]. One of these genes, TRAIL, is not only involved in cell death but also in the IFN signaling pathway [Kumar-Sinha et al., 2002]. Altogether, these results indicate and confirm that even though they can differentiate, HaCaT cells, as any keratinocyte, remain activated and partly correspond to the "contracted" keratinocytes described by Freedberg et al. [2001]. These results also suggest that growth factors and cytokines may act as differentiation promoting factors in keratinocyte cell culture.

Comparison of HaCaT to Normal Keratinocytes

Data obtained here on HaCaT cells using cDNA microarrays were compared to the Danish Centre for Human Genome database (http://proteomics.cancer.dk/jecelis/human_data_select.html). Celis and his colleagues induced the differentiation of primary keratinocytes by a switch in culture media from low calcium KGM to high calcium DMEM medium. We observed that among the 171 genes (see Table II) which were shown here to be regulated in our conditions, 54 were regulated in the same way as described in their previously established expression patterns. Only four transcripts (microglobulin, HRF, cathepsin D, and phorbolin I) were regulated in an opposite way [Olsen et al., 1995]. These differences may be in part due to differences in keratinocyte cell culture conditions and to the fact that we measured the steady level of gene expression whereas Celis and co-workers analyzed the ^{35}S labeled newly synthesized proteins by 2D electrophoresis.

Another aspect of our study concerns the protein analysis using 2D electrophoresis followed by MALDI mass spectrometry, which allowed us to identify 15 proteins regulated in differentiated HaCaT cells. Eight of these proteins were already known to be involved in keratinocyte differentiation, thus confirming data previously described in the Danish kerati-

TABLE III. Overview of Microarrays Data and Comparison of the Data Obtained in the Present Study to Previously Published Data

	New in keratinocyte and differentiation	New in differentiation	Known in keratinocyte and differentiation	Total
Up regulated genes	16	30	43	89
Down regulated genes	17	39	26	82

nocyte database. Seven other proteins, which have never before been reported to be regulated in this process, are described here as being differentially expressed (see Table I).

Even though our study focused on HaCaT cells, some data were assessed in primary cultures of human keratinocytes or in healthy human epidermis. RT-PCR analysis revealed that 31 of the 33 transcripts newly described in HaCaT cells (Table III) were also expressed in primary keratinocytes (Fig. 2A). Furthermore, twenty two of the later genes were differentially expressed in the same manner in HaCaT cells as in primary keratinocytes (Fig. 2B). Finally, the localization of four studied proteins in human epidermis correlated with results obtained using HaCaT cells (Fig. 3).

Altogether these observations tend to indicate that, under our differentiation conditions, HaCaT cells can be useful in the study of the differentiated states of keratinocytes since most regulations were in correlation between HaCaT keratinocytes and primary culture keratinocytes. Differences between this cell line and primary keratinocytes studies should be further investigated.

CONCLUSION

The use of cDNA microarrays combined with a proteomic approach allowed us to identify genes and proteins regulated in differentiated HaCaT keratinocytes, some of which have been confirmed in primary keratinocytes. For example, stathmin, which was found to be regulated in differentiated HaCaT cells by 2D and microarrays analysis, was confirmed in normal keratinocytes in culture using RT-PCR, in healthy skin by immunohistochemistry and could thus be proposed as a new marker of human keratinocytes.

Using data described in this study, a global view of HaCaT keratinocyte differentiation in culture can be drawn. Cell cycle was arrested and carbohydrate metabolism was down-regulated while cytokine signaling, cell adhesion, and cell immunity were up-regulated. Even if not all transcripts and proteins found to be regulated in our study can be easily related to the differentiation process, these results open new paths towards its understanding. Further experiments are under progress in order to investigate the role of some of these pathways in epidermal differentiation.

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