

THE ROLE OF D-TYPE CYCLINS IN HUMAN KERATINOCYTE CELL  
CYCLE REGULATION AND IN THE PATHOGENESIS OF PSORIASIS

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## Publications directly related to the subject of the dissertation

- I. **Belső N**, Széll M, Pivarcsi A, Kis K, Kormos B, Kenderessy SzA, Dobozy A, Kemény L, Bata-Csörgő Zs: Differential expression of D-type cyclins in HaCaT keratinocytes and psoriasis. *J Invest Dermatol* 128:634-42, 2008  
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- II. **Belső N**, Széll M, Pivarcsi A, Kis K, Kormos B, Kenderessy Szabó A, Dobozy A, Kemény L, Bata-Csörgő Zs: A D3 vitamin antipszoriaticus hatásának támadáspontjai lehetnek a pikkelysömörben kórosan kifejeződő D típusú ciklinek. *Bőrgyógy Vener Szle*, 84: 3-9, 2008
- III. **Belső N**, Bebes A, Kormos B, Széll M, Kemény L, Bata-Csörgő Zs: The combined silencing of D type cyclins results in S phase blockade and aberrant mitosis in HaCaT keratinocytes. Manuscript in preparation

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- I. Pivarcsi A, Koreck A, Bodai L, Széll M, Szeg Cs, **Belső N**, Kenderessy Szabó A, Bata Csörgő Zs, Dobozy A, Kemény L: Differentiation-regulated expression of Toll-like receptors 2 and 4 in HaCaT keratinocytes. *Arch Dermatol Res* 296:120-4, 2004  
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- II. Nagy N, Németh I B, Szabad G, Szolnoky Gy, **Belső N**, Dobozy A, Kemény L, Széll M: The altered expression of SDC4 in the uninvolved skin of venous leg ulcer patients may predispose to venous leg ulcer. *Wound Repair Regen*. In press

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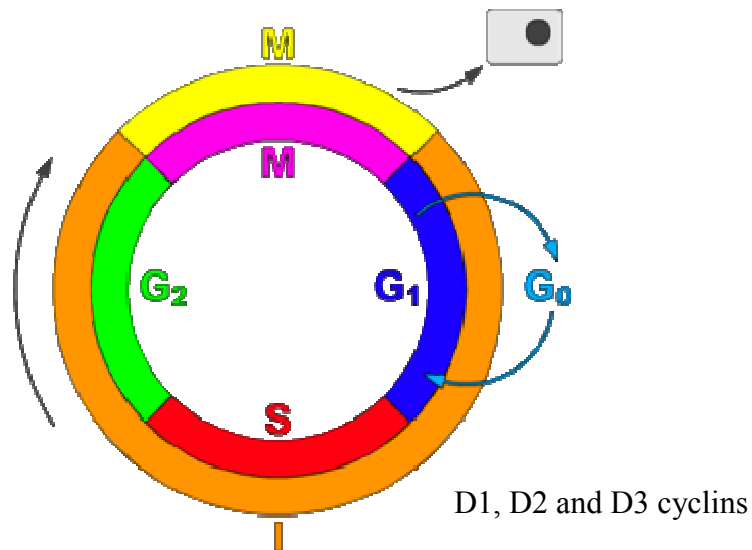
## List of abbreviations

ANOVA	analysis of variance
BSA	bovine serum albumin
FBS	fetal bovine serum
K1/K10	keratin1/keratin10
FITC	fluorescein isothiocyanate
mAb	monoclonal antibody
MFI	mean fluorescence intensity
PBS	phosphate buffered saline
PBSF	PBS with FBS
real-time RT-PCR	real—time reverse transcription-polymerase chain reaction
SD	standard deviation
SEM	standard error of mean
siRNA	RNA silencing
VDR	Vitamin D receptor

## 1. Introduction

The cell cycle regulatory system is based upon cyclin dependent protein kinases (Cdks). The activity of these kinases oscillates during cell cycle progress leading to phosphorylation of the cell cycle regulating proteins. This activity is regulated by several enzymes and proteins, most importantly by cyclins.

D-type cyclins are one key components of the cell cycle machinery<sup>34</sup>. Three D-cyclins - cyclin D1, D2 and D3 - operate in mammalian cells and play a major role in the positive regulation of G1 progression<sup>14</sup>. Enforced overexpression of D-type cyclins can shorten the G1 interval in cultured cells<sup>22;32</sup>. Following mitogen stimulation of quiescent cells, genes encoding D-type cyclins get activated at the beginning of the G1 phase<sup>15</sup>, and then the produced D-type cyclins bind to Cdk4 or Cdk6 to activate their kinase activity<sup>27;28</sup>. Microinjected antibodies or antisense constructs directed against D-type cyclins can interfere with G1 phase progression, without affecting other cell cycle intervals<sup>2;32</sup>.



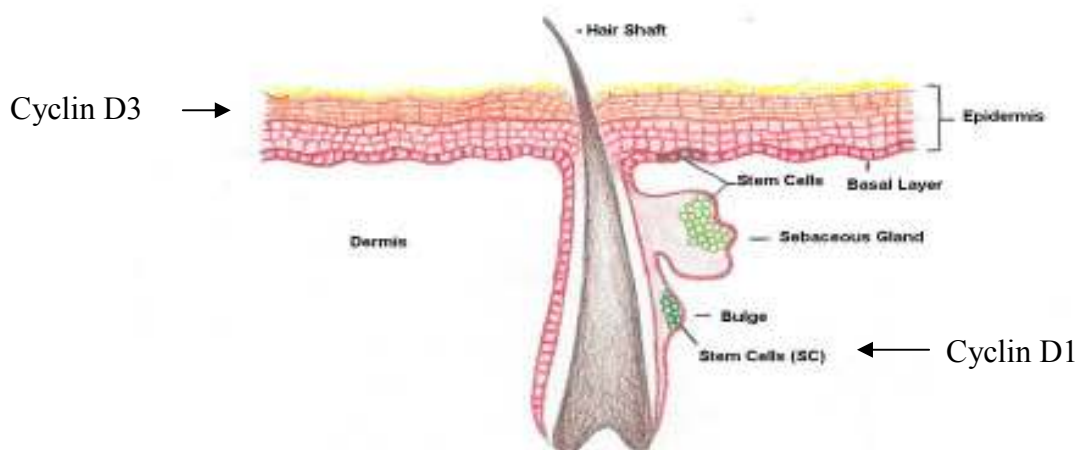
**Fig. 1.** Cell cycle regulation by D cyclins

These three proteins are encoded by separate genes located on different chromosomes, but they show significant amino acid similarities, suggesting that they arose from a common primordial ancestor gene<sup>20;43</sup>. In the developing skin of mice,

cyclin D1 is present in keratinocytes and is absent from developing hair follicles, while cyclin D2 exhibits an opposite pattern of expression <sup>1</sup>. In mice, cyclin D1 localizes to the proliferative layers in stratified squamous epithelia and in columnar gastrointestinal epithelium, whereas cyclin D3 is present in the adjacent compartments where differentiation takes place <sup>3</sup>.

### *1.1 The role of D-type cyclins in keratinocyte cell cycle regulation*

Quiescent (G0 phase) basal (keratin1/keratin10 negative) K1/K10<sup>-</sup> keratinocytes freshly isolated from human skin express D1, but not D2 type cyclin, when they almost synchronously traverse from G0-G1 into an initial cell cycle as a result of a wound response to disaggregation and in vitro culturing. As keratinocytes enter subsequent cell cycles, D1 expression disappears and D2 cyclin becomes clearly detectable. The differential expression of cyclin D1 and D2 in keratinocytes suggests specific functions for D-type cyclins and provides direct evidence that the G0-G1-S progression of quiescent keratinocytes is distinct from the G1-S traverse of already cycling cells <sup>4</sup>. Recently it was shown that cyclin D1-mediated proliferation predominantly occurs in the immediate progeny of the stem-cell-rich bulge cells in human hair follicle, indicating that cyclin D1 may be important for cells to exit the stem cell compartment <sup>44</sup>. On the other hand, cyclin D3 was reported to accumulate to high levels in the stratified squamous epithelial layers of postreplicative, differentiating cells in mice <sup>3</sup>.



**Fig. 2.** Expressions of cyclin D1 and D3 in the skin

The HaCaT keratinocyte cell line is a spontaneously transformed human epithelial cell line derived from a histologically normal skin specimen <sup>42</sup>. Despite the altered and unlimited growth potential, HaCaT cells, similarly to normal keratinocytes, form an orderly structured and differentiated epidermal tissue, when transplanted onto nude mice. They exhibit a number of chromosomal aberrations characteristic for skin SCCs but lack the gain of 11q, are non-tumorigenic, and still possess the ability to form a normally differentiated epidermis-like epithelium under appropriate conditions <sup>42</sup>. HaCaT cells are commonly used for gene transfection studies or as a model system to study hyperproliferative skin diseases such as psoriasis, or to study cancer development <sup>17;42</sup>.

Previously it has been described that cyclin D1 overexpression alters the proliferation and differentiation behavior of HaCaT cells in organotypic co-cultures, while the epithelia appeared undifferentiated, lacking morphologically distinct cell layers and signs of cornification <sup>9</sup>. In HaCaT epithelia as in the epidermis in situ proliferation is restricted to the basal layer <sup>12</sup>. In the cyclin D1 overexpressed HaCaT cell-organotypic co-culture BrdU-positive nuclei were only seen in the basal layer.

In our study we have investigated the expression of D-type cyclins both at the mRNA and protein levels in synchronized HaCaT keratinocytes to better understand their function in human keratinocytes.

### *1.2 Functions of D-type cyclins beside cell cycle regulation*

Data suggest that D-type cyclins are involved in a large complexity of networks of interactions and play some new roles besides acting as Cdk partners. Cyclin D1 regulates cellular metabolism, fat cell differentiation and cellular migration <sup>18</sup>, cyclin D2 plays a role in macrophage activation <sup>39</sup> and spermatogonial differentiation <sup>7</sup>, while cyclin D3 has a role in the promotion and maintenance of the terminally differentiated state of some cell types <sup>3</sup>.

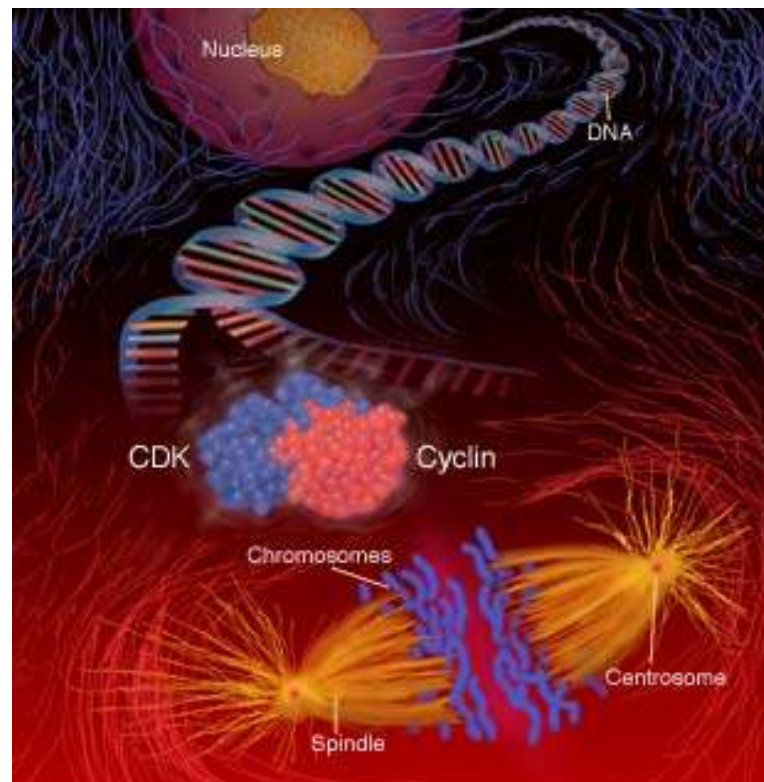
Knockout experiments in murine models also suggest that the function of different D cyclin subtypes is not entirely redundant. Knocking out the different D cyclin isotypes in mice results in different phenotypes: „Cyclin D1 only” mice develop severe megaloblastic anaemia, „cyclin D2 only” mice present neurological abnormalities, and „cyclin D3 only” mice lack normal cerebella <sup>14</sup>. Mice lacking cyclin D1 display hypoplastic retinas, underdeveloped mammary glands and present



developmental neurological abnormalities. At the same time knock in strain of mice expressing cyclin D2 in place of D1 shows nearly normal development of retinas and mammary glands, and D2 can partially replace the function of cyclin D1 in neurological development <sup>11</sup>.

In HeLa cells specific knockdown of cyclin D3 by small interfering RNA results in a delayed progression through G2 phase and mitosis. HeLa cells exit mitosis with a resultant increase in cells with multiple or micronuclei because chromosomes fail to migrate to the metaphase plate. These data indicate an important function for cyclin D3 activity in the S/G2 phase <sup>8</sup>. In bronchial epithelial (BEAS) cells the siRNA targeting of each D-type cyclin caused growth suppression. Multiple siRNA transfection targeting all the D-type cyclins resulted in cooperative growth suppression in these cells indicating that functional interactions are needed for efficient cell growth in BEAS cells

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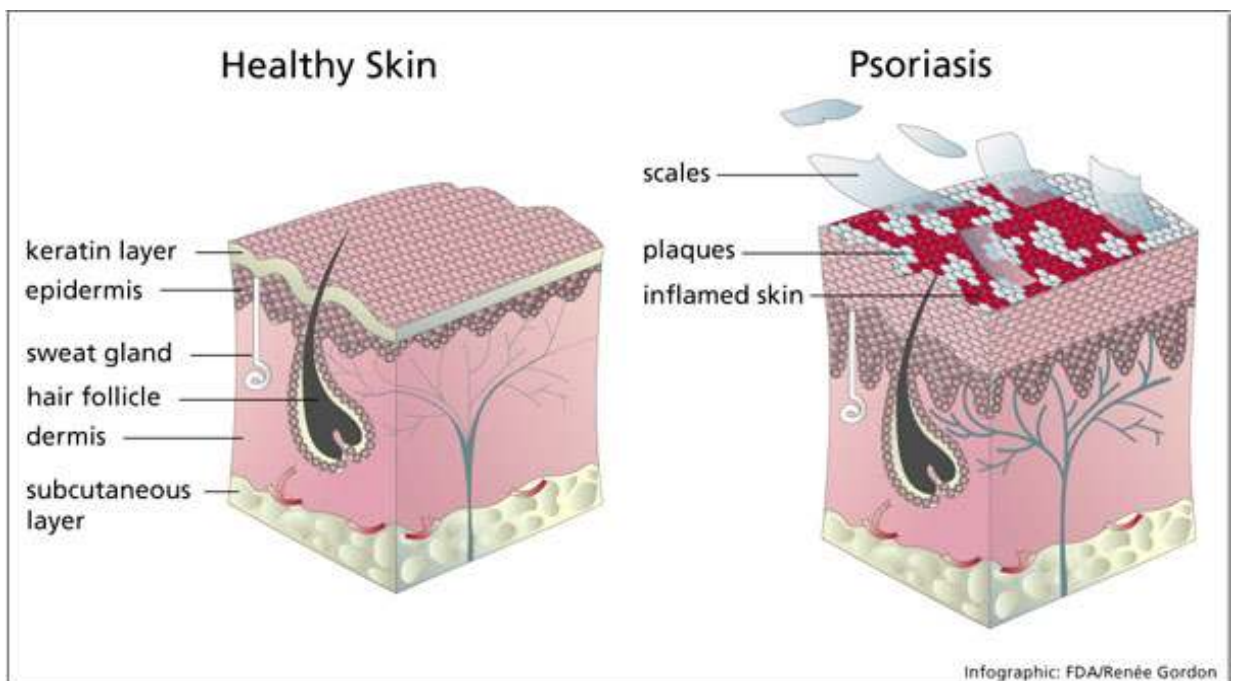
**Fig. 3.** Function for cyclin D3 activity in the S/G2 phase

To better understand the specific functions of D-type cyclins in HaCaT cells we have used multiple gene specific silencing. Our results indicate that in HaCaT keratinocytes, similar to HeLa cells, D-type cyclins are important for G2/M traverse and

normal mitosis. As long as two D-type cyclins function in these cells they exhibit unaltered morphology, proliferation and mitosis, indicating that they can partially replace each other in cell cycle regulatory functions.

### *1.3 Epidermal hyperproliferation and abnormal differentiation in psoriasis*

Psoriasis is considered to be a multifactorial inflammatory skin disease with distinct hyperproliferation of the normally quiescent basal keratinocyte population which contains the keratinocyte stem cells <sup>6</sup>. Although, there is strong evidence that dysregulated inflammation plays a pivotal role in disease development and maintenance, data indicate that epidermal abnormalities contribute to disease susceptibility. Fibronectin as a prominent component of extracellular matrix has been shown to increase cell cycle entry among uninvolved but not normal keratinocytes <sup>5</sup>. Concordantly,  $\alpha 5$  integrin the receptor of fibronectin, but not  $\alpha 2$  or  $\alpha 3$ , is overexpressed in the in vivo non-lesional psoriatic epidermis <sup>5;30</sup>. The role of abnormal  $\alpha 5$  integrin expression in psoriasis pathogenesis is further supported by the fact that mice with forced suprabasal integrin expression exhibit epidermal hyperproliferation, perturbed keratinocyte differentiation, and skin inflammation <sup>10</sup>.



**Fig. 4.** Psoriasis vs healthy skin

Because D-type cyclins are major sensors of cell cycle driving environmental signals we characterized the expression of D-type cyclins in psoriasis and examined the role of  $\alpha 5$  integrin in the regulation of cyclin D1 mRNA expression.

#### *1.4 The role of D3 vitamin in the therapy of psoriasis*

1,25-Dihydroxyvitamin D3 and its analogs represent candidate compounds for treatment of hyperproliferative diseases including psoriasis and diverse types of cancer. A major advantage of these reagents lies in the ability not only to halt proliferation, but also to induce differentiation or cell death<sup>36,41</sup>. The cell cycle arrest induced by 1,25-VD3 and its analogs has been investigated in tumor cells of leukemic, prostate, pancreatic and breast cancer origin, as well as in normal keratinocytes. The consensus view emerging from these studies identifies G1 phase as the major target of the observed cell cycle blockade. A series of studies found that D-type cyclins bind several transcription factors and regulate their activity. Cyclin D1 could form a specific complex with estrogen receptor (ER) and activate the transcription activity of the receptor in a Cdk4 and ligand independent manner. Cyclin D2 and D3 were also found to associate with the estrogen receptor to a lesser extent and showed a less pronounced effect than cyclin D1 on ER<sup>47</sup>. Vitamin D receptor (VDR) is a member of the superfamily of nuclear receptors for steroid hormones and Yian et al. showed that cyclin D3 interacts with VDR and regulates its transcription activity<sup>21</sup>.

We have shown in our previous experiments that D-type cyclins regulate the G1/S phase of the cultured primary keratinocyte cell cycle<sup>4</sup>. As psoriasis can be characterized with the hyperproliferation of keratinocytes we were interested in seeing whether the inhibitory activity of 1,25-Dihydroxyvitamin D3, which is used for the treatment of psoriasis, is related to its effect on the gene expressions of D-type cyclins. Here we have investigated the effect of 1,25-Dihydroxyvitamin D3 treatment on the expression of D-type cyclins at the mRNA level on synchronized HaCaT keratinocytes.

## 2. Aims

The aims of our study were:

- to characterize the expression pattern of D-type cyclins in synchronized HaCaT keratinocytes.
- to investigate the functions of D-type cyclins in keratinocytes
- to compare the expression of D-type cyclins in healthy, psoriatic lesional and non-lesional epidermis.
- to determine whether the proliferation-related  $\alpha 5$  integrin regulates the expression of cyclin D1
- to assess the effect of D3 vitamin on the expression of D-type cyclins

## 3. Materials and Methods

### *3.1 Cell culture*

#### *3.1.1 HaCaT keratinocytes*

Cells of the spontaneously transformed human keratinocyte cell line HaCaT (kindly provided by N.E. Fusenig, Heidelberg, Germany) were cultured in high-glucose DMEM (Invitrogen Corporation, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corporation), 1% 200 mM L-glutamine and 1% antibiotic/antimycotic solution (Sigma) (complete DMEM) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The medium was changed every 2 days.

### *3.2 Human tissue samples*

Shave biopsies were taken from uninvolved and involved skin of psoriatic patients from the buttock area. Control skin biopsies from the breast and the stomach area were obtained from healthy individuals undergoing plastic surgery. After removal of the subcutaneous tissue, skin biopsies were incubated overnight at 4°C in Dispase solution (grade II; Roche Diagnostics, Mannheim, Germany). On the following day, the epidermis was separated from the dermis. All tissue samples were taken with the patient's informed consent and the approval of the local ethics committee. The study was conducted according to the Declaration of Helsinki Principles.

### *3.3 Synchronization procedure*

HaCaT keratinocytes were synchronized and cultured as described previously<sup>31</sup>. Samples were collected 0, 12, 24, 36, 48, 72, 96 and 168h following the end of the synchronization process.

### *3.4 Real-time RT-PCR*

Total RNA was isolated from the epidermis obtained from shave biopsies after Dispase digestion and from HaCaT keratinocytes using TRIzol™ reagent (Gibco, Invitrogen,

Carlsbad CA, USA) following the instructions of the manufacturer. RNA concentration was determined by the A260 values. First strand cDNA was synthesized from 1 µg of total RNA using the iScript™ cDNA Synthesis Kit from Bio-Rad (Hercules, CA). Real-time RT-PCR experiments were performed to quantify the relative abundance of each mRNA by using the iCycler IQ Real-Time PCR machine of BioRad (Hercules, CA). Primers specific for cyclin D1 (Cat. Hs 00277039), D2 (Cat. Hs 00153380) and D3 (Cat. Hs 00236949) were purchased from Applied Biosystems (Foster City, CA). The abundance of each gene of interest was normalized to the expression of 18S ribosomal RNA of each examined sample. The primers for 18S were: 18S RNA forward: CGG CTA CCA CAT CCA AGG AA, 18S RNA reverse: GCT GGA ATT ACC GCG GCT, 18S RNA TaqMan probe: TexRed - TGC TGG CAC CAG ACT TGC CCT C - BHQ-1 (Integrated DNA Technologies, Coralville, IA).

### *3.5 Flow cytometry*

The epidermis was separated from the dermis as described previously. Epidermal and HaCaT keratinocyte suspensions were prepared using trypsin (0.25% for epidermal and 0.025% for HaCaT cells). Cells were fixed in -20°C cold 70% ethanol (Reanal, Budapest, Hungary) and kept at -20°C for at least overnight before staining. The following monoclonal antibodies were used: Purified Mouse Anti-Human Cyclin D1, Purified Mouse Anti-Human Cyclin D2 and Purified Mouse Anti-Human Cyclin D3 at 1:200 dilution, all from BD Pharmingen (San Diego, CA), mouse IgG1, mouse IgG2a and mouse IgG2b at the same concentrations served as isotype controls (all from Sigma, Steinheim, Germany). Secondary antibodies were anti-mouse IgG1, IgG2a and IgG2b coupled to fluorescein isothiocyanate (FITC), used at 1:50 and mouse IgG2b at 1:500 dilutions all from BD Pharmingen (San Diego, CA). Analysis was carried out using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) and CellQuest software (Becton Dickinson). Results were expressed either as the geometric mean fluorescence intensity (GeoMean) calculated by subtraction (GeoMean of the antigen specific antibodies stained cells - GeoMean of isotype-matched control antibodies stained cells), or as % positive cells.

### 3.6 Gene silencing

HaCaT keratinocytes were contact inhibited for 1 day and serum starved for 5 days. Cells were then harvested and nucleofected with cyclin D1, D2 and D3 siRNAs by Nucleofector™ II (Amaxa Biosystems, Koeln, Germany) using V-Kit optimized for HaCaT keratinocytes (VCA-1003, Amaxa Biosystems, Koeln, Germany) in suspensions and then plated into 12 well tissue culture plates and cultured in antibiotic free medium containing 10% fetal bovine serum and incubated at 37°C. Cyclin D1 siRNA (sc 29286), Cyclin D3 siRNA (sc 35136) siControl RNA (sc 36869) all from Santa Cruz Biotechnology, INC (Santa Cruz, California) and CCND 2 (Cyclin D2) SMARTpool siRNA (M 003211) from Upstate (Billerica, USA) were used following the instructions of the manufacturer. Samples were taken at 24, 48 and 72 h following the passage of the cells. Control siRNA is a non-targeting 25 nt siRNA designed as a transfection control. Total RNA was isolated using TRIzol™ reagent (Invitrogen, Carlsbad CA, USA). Real-Time RT-PCR was carried out as described above to quantify the mRNA expression of cyclin D1, D2 and D3. The transfection efficacy was detected using a plasmid encoding the enhanced green fluorescent protein eGFP (Amaxa Biosystems, Koeln, Germany) 48 hours post transfection by flow cytometry.

### 3.7 MTT (*Thiazolyl Blue Tetrazolium Bromide*) assay

HaCaT keratinocytes were contact inhibited for 1 day and serum starved for 5 days. Cells were then trypsinized and nucleofected in cell suspension with cyclin D1 siRNA. HaCaT keratinocytes were seeded into wells of 96-well plates directly after transfection at a density of  $5 \times 10^3$  cells/well and grown in 200 µl complete high glucose DMEM medium for 24, 48, 72, 96, and 168 hours. MTT assays were carried out as described previously<sup>35</sup>.

### 3.8 Immunohistochemical staining

Formalin-fixed, paraffin embedded tissue sections were dewaxed. Slides were placed in a slide rack and immersed into 500 ml of 10 mM citric acid buffer (pH 6.0). After incubation in a microwave oven for 30 min at 700 W, during which the incubation solution boiled for about 25 min, slides were rinsed with TBST (Tris buffered saline, Sigma, Steinheim, Germany, containing 0.1% Triton-X, Reanal, Budapest, Hungary)

for 15 min. Non-specific staining was prevented by pre-incubation with 0.5% bovine serum albumin diluted in TBST (Tris buffered saline, Sigma, Steinheim, Germany, containing 0.1% Triton-X, Reanal, Budapest, Hungary). Cells were then incubated overnight at 4°C in a humid chamber with the primary antibodies. Purified monoclonal antibodies to cyclin D1, cyclin D3 (NeoMarkers, Fremont, CA) and cyclin D2 (Fitzgerald Industries Int., Inc., Concord MA, USA) were applied at 1:200, 1:25 and 1:2 dilutions respectively, rabbit IgG (NeoMarkers, Fremont, CA), mouse IgG1 and mouse IgG2a (Sigma, Steinheim, Germany) were used for isotype control stainings. Slides were then incubated with a biotinylated secondary antibody (anti-rabbit IgG for cyclin D1, anti-mouse IgG for cyclin D2 and D3 all at 3.3 µg/ml concentration), followed by incubation with horse radish peroxidase-conjugated streptavidin (Vectastain ABC Kit, Vector, Burlingame, USA), and finally peroxidase activity was detected using 3,3-amino-9 ethylcarbazole (AEC, Sigma) as a substrate. Slides were counterstained with hematoxylin (Sigma, Steinheim, Germany). Tissue staining was visualized using a Zeiss Axio Imager microscope and photographed using a PixelINK digital camera.

### *3.9 α5 integrin blocking assay*

1x10<sup>6</sup> synchronized HaCaT keratinocytes were seeded into 25 cm<sup>2</sup> cell culture flasks (Sarstedt, Germany). 5 hours after seeding, when the cells already attached to the surface of the culture flask, an α5 integrin neutralizing purified mouse anti-human monoclonal antibody (CD49e, no azide-low endotoxin, BD Pharmingen, San Diego, USA) was added to the cells at 20 µg/ml final concentration. Mouse IgG1 (BD Pharmingen, San Diego, USA) at identical concentration was used for isotype control. HaCaT keratinocytes were incubated with the antibody for 7 hours. Samples were collected before (0 hours) and 12 hours following the seeding of the cells. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad CA, USA) following the instructions of the manufacturer. Samples were analyzed by real-time RT-PCR to quantify the mRNA expression of cyclin D1, D2 and D3.

### *3.10 Fluorescent immunocytochemistry*

HaCaT keratinocytes were contact inhibited for 1 day and serum starved for 5 days. Cells were then trypsinized and nucleofected in cell suspension with cyclin D1, D2 and D3 siRNAs. HaCaT keratinocytes were seeded onto 8 Chamber Polystyrene Vessel



Tissue Culture Treated Glass Slides (Falcon CultureSlide, Becton Dickinson Labware Europe, France). Cultured cells on slides were fixed in 2% paraformaldehyde. Non specific staining was prevented by pre incubation with 0.5% bovine serum albumin diluted in TBST (Tris buffered saline, Sigma, Steinheim, Germany, containing 0.1% Triton X, Reanal, Budapest, Hungary). Cells were then incubated overnight at 4°C in a humid chamber with the primary antibodies. Purified monoclonal antibodies to cyclin D1, cyclin D3 (NeoMarkers, Fremont, CA) and cyclin D2 (Fitzgerald Industries Int., Inc., Concord MA, USA) were applied at 1:200, 1:25 and 1:1 dilutions respectively, rabbit IgG (NeoMarkers, Fremont, CA), mouse IgG1 and mouse IgG2a (Sigma, Steinheim, Germany) were used for isotype control stainings. Slides were then incubated with fluorescent conjugated secondary antibodies (Alexa Fluor 488 goat anti rabbit IgG for cyclin D1 and Alexa Fluor 647 goat anti mouse IgG for cyclin D2 and D3, all at 2.5 µg/ml final concentration), followed by incubation with 4,6 Diamidino 2 phenylindole dihydrochloride (DAPI, Sigma, Steinheim, Germany at 1 µg/ml final concentration). Cell staining was visualized using a Zeiss Axio Imager microscope and photographed using a PixelFLY (PCO) digital camera.

### *3.10 Apo 2.7-PE staining*

HaCaT keratinocytes were contact inhibited for 1 day and serum starved for 5 days. Cells were then harvested and nucleofected with cyclin D1, D2 and D3 siRNA in suspensions and then plated into 12 well tissue culture plates and cultured in antibiotic free medium containing 10% fetal bovine serum. Cells were harvested by trypsinization and washed in PBS. Cell density was adjusted to  $1 \times 10^6$  cells/ml. The pellet was resuspended in 100 µl cold 4°C 100 µg/ml digitonin in PBSF (PBS with 2.5% FBS). Cells were washed in 4°C PBSF and the pellet was resuspended in 20 µl of Apo 2.7 PE (Coulter, Immunotech, Marseille, France) and 80 µl of PBSF and incubated for 15 min in the dark at RT. After washing, cells were resuspended in 1 ml of PBSF. Analysis was carried out using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) and CellQuest software (Beckton Dickinson).

### *3.12 Propidium iodide DNA staining*

HaCaT keratinocytes were harvested, nucleofected and cultured as described above. Cell density was adjusted to  $1 \times 10^6$  cells/ml. The pellet was resuspended in 70% cold (20°C) ethanol and fixed for at least 24h at 20°C. After fixation, cells were centrifuged and washed in PBS. Cells were then resuspended in 500 µl PI/RNase staining buffer (containing 50 µg/ml PI and 100 U/ml RNase A from BD) and left for 30 min at room temperature. The samples were analysed using a FACS Calibur machine (Becton Dickinson) and evaluated by Modfit software (Verity Software House, Topsham, ME).

### *3.13 Immunocytochemistry*

HaCaT keratinocytes were treated, fixed and stained with primary antibodies as described above. Purified monoclonal antibodies to cytokeratin 1 and 10 and integrin alpha 5 (Abcam, Cambridge, UK) were applied at 1:800 and 1:500 dilutions respectively, mouse IgG1 and mouse IgG2b (Sigma, Steinheim, Germany) were used for isotype control stainings. Slides were then incubated with biotinylated secondary antibody (anti mouse IgG for both cytokeratin 1 and 10 and alpha 5 integrin were applied, all at 3.3 µg/ml concentration) for 1h at room temperature, followed by incubation with horse radish peroxidase conjugated streptavidin for 1h at room temperature (Vectastain ABC Kit, Vector, Burlingame, USA), and finally peroxidase activity was detected using 3,3 amino 9 ethylcarbazole (AEC, Sigma) as a substrate. Slides were counterstained with hematoxylin (Sigma, Steinheim, Germany). Tissue staining was visualized using a Zeiss Axio Imager microscope and photographed using a PixeLINK digital camera.

### *3.14 1,25-dihydroxyvitamin D3 treatment of the cells*

Synchronized HaCaT keratinocytes were treated with 1,25-Dihydroxyvitamin D3 at a  $10^{-7}$  M concentration. Control cells were treated with the vehicle – ethanol at identical concentration. Samples were collected 42 hours after D3 vitamin treatment, at the time cells were 48 hours after release from cell quiescence.

### *3.15 Data presentation*

Gene expression results were expressed as fold increases over control values. Data were presented as mean +/- Standard Deviation (SD) for n experiments. Flow cytometry data were expressed either as % over isotype values or as the geometric mean fluorescence intensity (GeoMean) calculated by subtraction of the GeoMean of isotype stained cells from the GeoMean of the specific antibody stained cells. Data were compared using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test to determine statistical differences after multiple comparisons (SPSS, SPSS Inc., Chichago, IL). Data from the  $\alpha 5$  integrin blocking assay were compared using the Mann-Whitney U test. Probability values of less than 0.05 for both parametric and non-parametric tests were considered significant.

## 4. Results

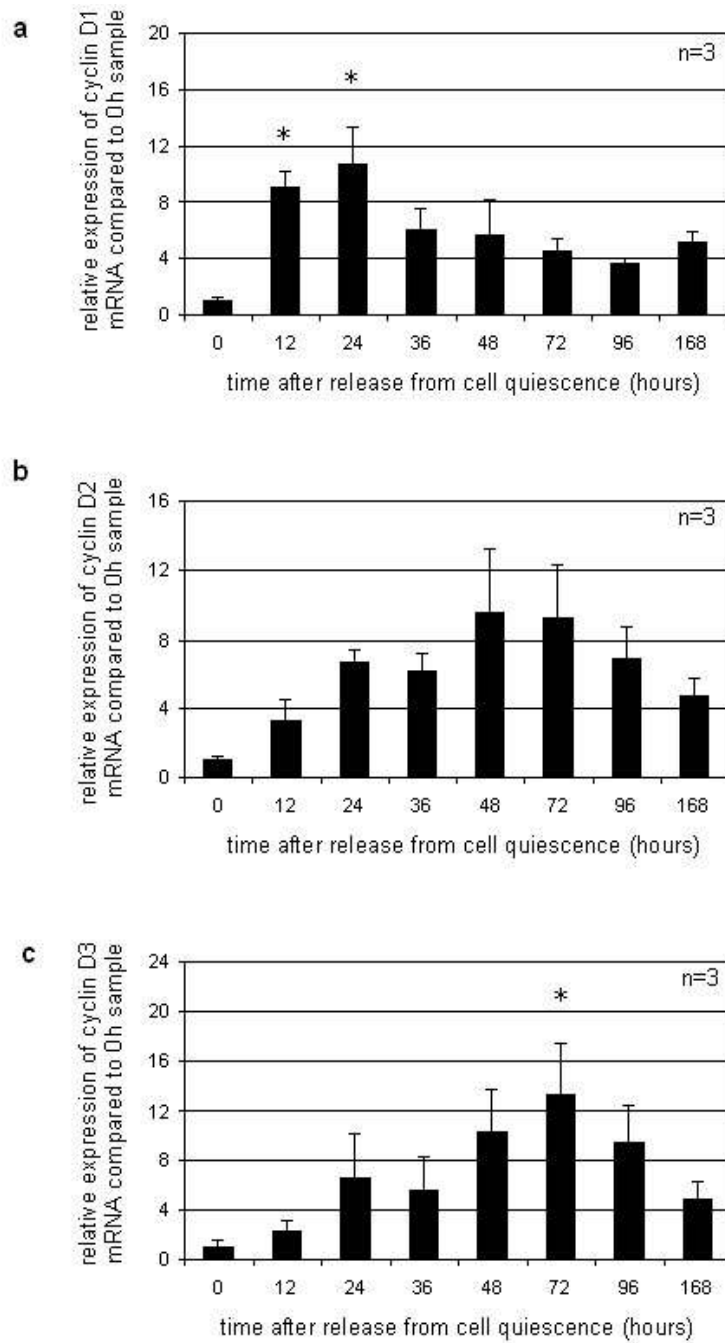
### *4.1 Examination of D-type cyclin expression in synchronized HaCaT cells*

#### ***4.1.1 The expression of cyclin D1 both at the mRNA and protein levels increases in cells after they leave the G0 quiescent phase and cyclin D2 and D3 expression occurs in already cycling cells***

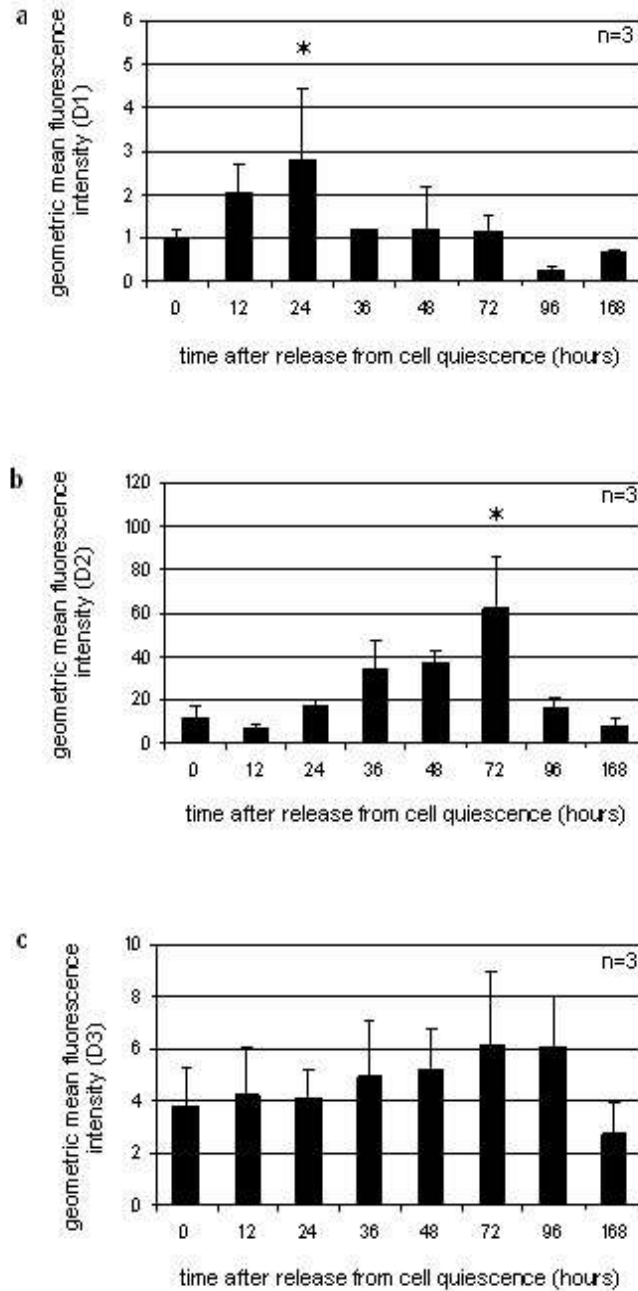
To characterize cell cycle specific D cyclin expression in HaCaT keratinocytes we used synchronized cell cultures (n=3). An almost complete cell cycle withdrawal can be achieved in these cultures by contact inhibition and serum starvation, as we have previously demonstrated by cell cycle analysis of PI stained cells <sup>31</sup>. Quiescent cells at the time of release from growth inhibition express almost undetectable cyclin D1 mRNA. A gradual increase in cyclin D1 mRNA can be detected in the cells between 24 and 72 h after release from cell quiescence, closely followed by increasing number of cells entering S phase. Cyclin D1 expression is preceded by the increase of  $\alpha 5$  integrin mRNA in the cells, at the same time mRNAs for the early differentiation markers K1/K10 substantially decrease. By the end of a one-week culture period, keratin expression reoccurs in the cells parallel with suppression of  $\alpha 5$  integrin and cyclin D1 expression and with reduction of proliferation <sup>31</sup>. The expression of cyclin D1, D2 and D3 at the mRNA levels were determined in synchronized HaCaT keratinocyte cultures at 0, 12, 24, 36, 48, 72, 96 and 168 hours following the end of the synchronization process, using real-time RT-PCR. The serum starved, contact inhibited HaCaT keratinocytes, similar to our previous observation, expressed a very low level of cyclin D1 mRNA (0h sample), which increased significantly with time after passaging and serum readdition. The highest level of cyclin D1 mRNA expression (10.7 fold increase compared to 0h samples) was detected 24 hours after release from cell quiescence when cells started to proliferate (Fig 5 a). Cyclin D2 and D3 mRNA also showed low levels of expression in quiescent cells (0 hours). Cyclin D2 and D3 mRNA levels gradually increased in the cells after release from cell quiescence, but their peak expression occurred at 48-72 hours in already proliferating cells (Fig 5 b and c). Both cyclin D2 (Fig 5 b) and cyclin D3 (Fig 5 c) mRNA expressions showed maximum levels at 48 and 72 hours (9.62 and 9.23 fold and 10.22 and 13.22 fold increases, respectively, compared

to the 0h samples). As cell cultures became confluent mRNA levels for all cyclins have decreased.

The levels of cyclin D1, D2 and D3 protein expression showed similar kinetics to the mRNA expressions when analyzed by flow cytometry (n=3). Cyclin D1 expression was low in serum starved quiescent cells (5.74 +/- 4.01% of all cells expressed cyclin D1) (Fig 6 a), at the same time cyclin D2 and D3 proteins showed higher expressions (41.25 +/- 6.31% cells were positive for D2 and 27.32 +/- 1.38% were positive for D3 cyclin) (Fig 6 c). Both D1 and D2 cyclin expression increased considerably after release from cell quiescence (Fig 6 a, b). The highest level of cyclin D1 protein expression (2.82 fold increase in geometric mean fluorescence intensity /GeoMean/ compared to the 0h samples) was detected 24 hours after release from cell quiescence (28.97 +/- 3.3% cells were positive for cyclin D1) (Fig 6 a). Cyclin D2 expression peaked later at 72 hours in the already highly proliferative cultures (5.17 fold increase in GeoMean at 72h compared to 0h samples, Fig 6 b) where 83.41 +/- 3.6% of the cells showed positive staining. For D3 protein expression only a slight increase was detected in the cells at 72 and 96 hours compared to the 0h level (1.6 and 1.57 fold increase in GeoMean), however, 66.08 +/- 6.67% cells were positive for cyclin D3) (Fig 6 c).



**Fig. 5.** Cyclin D1, D2 and D3 mRNA expression in synchronized HaCaT keratinocytes. HaCaT keratinocytes (n=3) were arrested by serum deprivation and contact inhibition, then stimulated to re-enter the cell cycle by passaging them into serum containing medium. Changes in the expression of cyclin D1, D2 and D3 mRNA were analyzed by real-time RT-PCR (a, b and c) at the times indicated after release from cell quiescence. Values are shown as relative expression compared to 0 h sample (mean  $\pm$  SD, \* $p < 0.05$ , Dunnett's test).



**Fig. 6.** Cyclin D1, D2 and D3 protein expression in synchronized HaCaT keratinocytes. HaCaT keratinocytes (n=3) were synchronized by contact inhibition and serum starvation (0h), and then released from cell quiescence by serum re-addition and passage. Flow cytometric analysis was used to detect the expression of cyclin D1 (a), D2 (b) and D3 (c) protein in the cells at the indicated times. The geometric mean fluorescence intensity (GeoMean) was calculated by subtraction of the GeoMean of isotype stained cells from the GeoMean of the specific antibody stained cells using CellQuest Software (mean +/- SD, \*p < 0.05, Dunnett's test).

## *4.2 Examination of the functions of D-type cyclins beside cell cycle regulation*

### ***4.2.1 Nor proliferation, neither cell morphology is affected by single cyclin D gene-specific silencing in HaCaT keratinocytes released from cell quiescence, but the combined silencing of D-type cyclins results in the appearance of large, multinucleated cells, S phase arrest and moderate growth suppression***

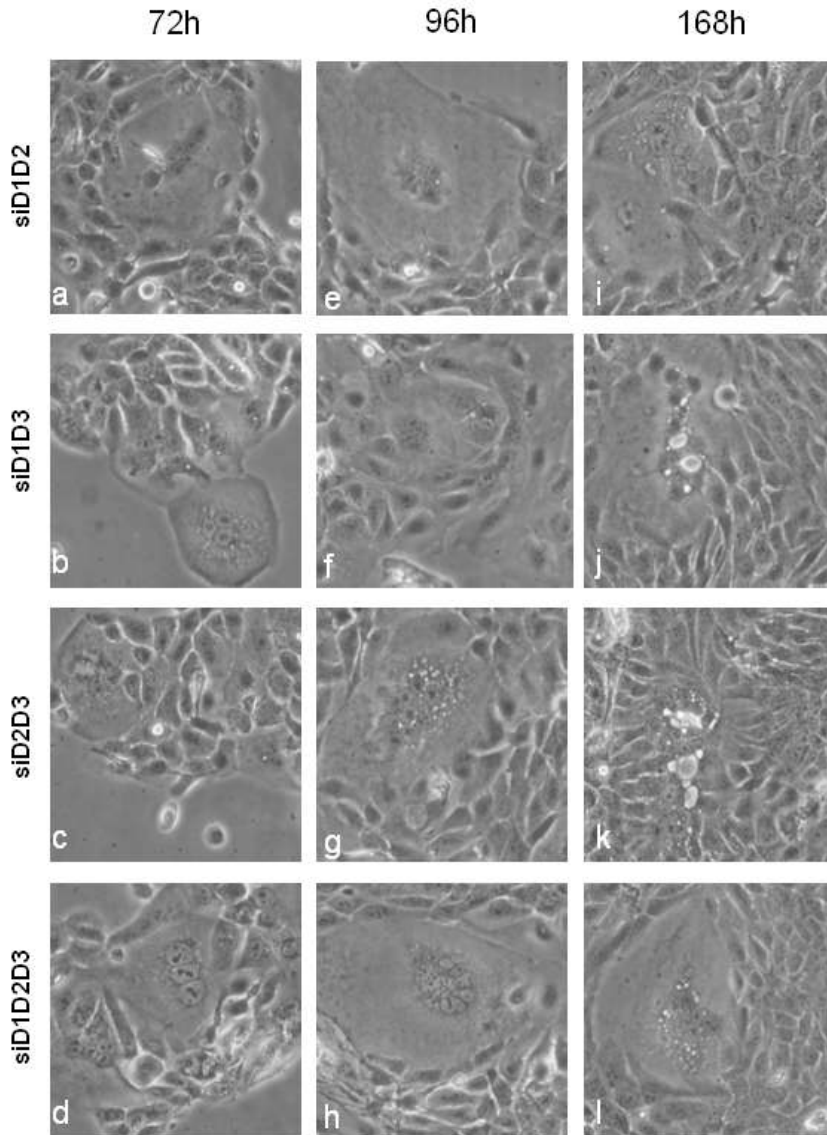
Next we addressed the question whether cyclin D1 function was essential for the quiescent HaCaT keratinocytes to proliferate after release from contact inhibition and serum starvation. HaCaT keratinocytes were contact inhibited for 1 day and serum starved for five days. Cells were then harvested and nucleofected with cyclin D1 siRNA in suspensions and then plated and cultured. Two independent experiments were carried out, both gave similar results. The transfection efficacy was 71.37 and 90.24%. We show the results of the second experiment where the transfection efficacy was 90.24%. Cyclin D1, D2 and D3 mRNAs were determined at 24, 48 and 72 hours after transfection. Control cultures were treated similarly and transfected with mock siRNA for cyclin D1 or not transfected. As expected, D1 siRNA significantly down-regulated cyclin D1 expression in the transfected HaCaT keratinocytes at 24, 48 and 72 hours following the plating of the cells. Silencing of D1 mRNA expression had no effect on the D2 and D3 mRNAs expressions. Cell numbers determined by MTT assay at the same time points were similar in all cultures. Cell and colony morphology in D1 cyclin silenced HaCaT cultures did not show any obvious difference compared to both controls (data not shown).

We also determined the role of D2 and D3 cyclins in the proliferation of HaCaT keratinocytes. HaCaT cells were treated as described before. Cells were nucleofected with cyclin D2 and D3 siRNAs in suspensions and then plated and cultured. Control cultures were treated similarly and transfected with mock siRNA for cyclin D2 and D3. As expected D2 and D3 siRNAs significantly down regulated cyclin D2 and D3 mRNA expressions in the transfected HaCaT keratinocytes. Silencing of D2 and D3 mRNAs expressions had no effect on the mRNA expressions of the remaining D-type cyclins. Cell numbers determined by MTT assay were similar in all cultures (data not shown).



Cell and colony morphology in D2 and D3 cyclins silenced HaCaT cultures did not show any obvious difference compared to the control cells (data not shown).

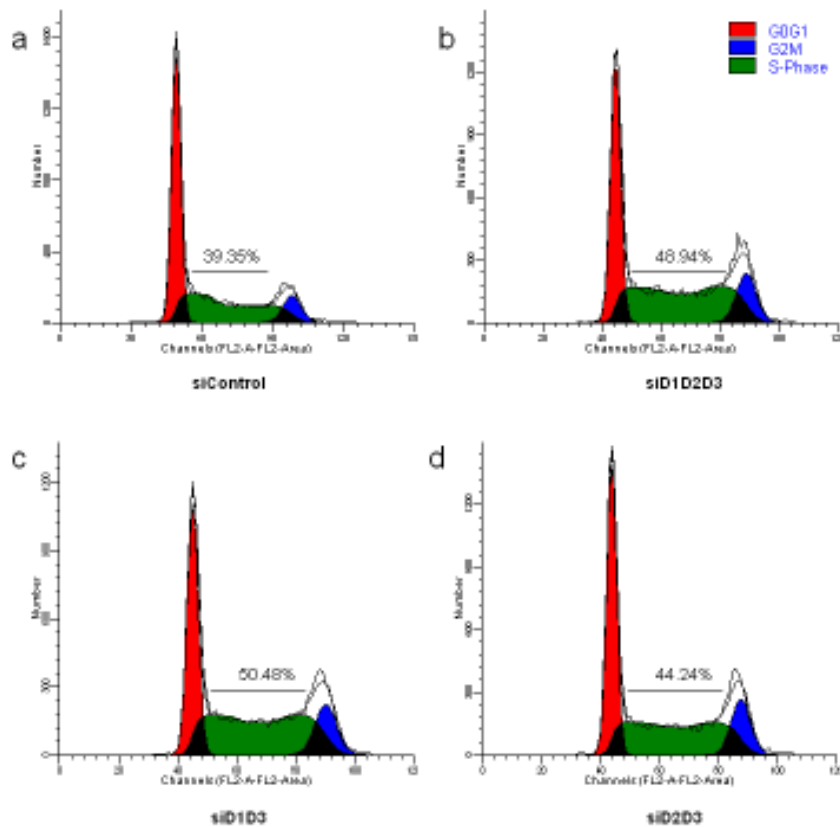
Next we addressed the question whether cells in which two or three D-type cyclins were silenced at the same time were able to keep their proliferative and morphological characteristics. HaCaT keratinocytes were contact inhibited for one day and serum starved for five days. Cells were then harvested and nucleofected with the combination of cyclin D1 D2, D1 D3, D2 D3 (double silenced) and D1 D2 D3 (triple silenced) siRNAs in suspensions and then plated and cultured. Control cultures were treated similarly and transfected with mock siRNA for D-type cyclins. The double and triple silencing of D-type cyclins resulted in remarkable downregulation of the silenced cyclin mRNAs. In double silenced cells there was no compensatory increase in the remaining, not silenced cyclin mRNA expression, in fact a small decrease was apparent relative to controls. Silencing resulted in the disappearance of the specific protein, while the remaining non silenced cyclin protein stayed detectable in the cells. 72 hours after nucleofection and release from cell quiescence large, multinucleated cells were apparent in all multiple D-type cyclin silenced cultures (Fig 7 a, b, c, d). In the next 48 hour we detected growing numbers of small vacuoles in the cytoplasm of these aberrant cells (Fig 7 e, f, g, h, i, j, k, l).



**Fig. 7.** Combined silencing of D-type cyclins by siRNAs results in the formation of large, multinucleated cells. Contact inhibited and serum starved HaCaT keratinocytes were nucleofected with the combination of cyclin D1 D2 (a, e, i), D1 D3 (b, f, j), D2 D3 (c, g, k) and D1 D2 D3 (d, h, l) siRNAs and Control siRNA and were cultured for 72, 96 and 168 hours. 72 hours after the combined silencing of D-type cyclins large, multinucleated cells were apparent in the cultures (a, b, c, d). During the subsequent 96 hours, both at 96 hours (e, f, g, h) as well as 168 hours (i, j, k, l) growing numbers of small vacuoles were detected in the cytoplasm of these cells. Obj. 20x

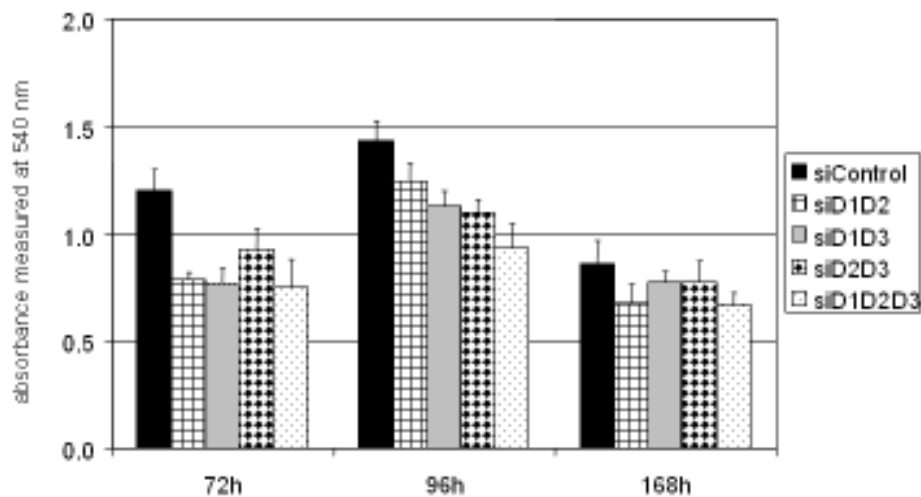
To determine the effect of multiple D-type cyclin silencing on cell cycle progression, propidium iodide DNA analysis was performed. Seventy two hours after nucleofection, at the time of the appearance of large multinucleated cells, we measured an increased

number of cells in S phase in the double and triple D cyclin silenced cells (Fig 8). 48.94% of cyclin D1 D2 D3, 50.48% of cyclin D1 D3, 44.24% of cyclin D2 D3 silenced and 39.35% of Control siRNA treated cells were in S phase (Fig 8 a-d). At later timepoints (96 and 168 hours) we have found the same tendency: at 96 hour 45.67% and at 168 hour 26.06% of the triple D cyclin silenced cells were in S phase compared to 40.81% at 96 hour and 15.91% at 168 hour of the Control siRNA treated cells (data not shown). This increase in S phase fraction, together with the detected growth suppression indicate that cells endure a G2/M block when silenced for two or three D-type cyclins. Flow cytometric analysis revealed no apoptotic effect on these cells as measured by Apo2.7 staining (data not shown).



**Fig. 8.** Combined silencing of D-type cyclins by siRNA results in S phase block. Contact inhibited and serum starved HaCaT keratinocytes were nucleofected with the combination of cyclin D1 D3 (c), D2 D3 (d) and D1 D2 D3 (b) siRNAs and Control siRNA (a) and were cultured for 72 hours. Cell cycle analysis was carried out using PI DNA staining. Data were acquired by a Fascalibur (BD) and DNA analysis was performed with Modfit software. Percentages of cells in S phase are indicated. Values shown are from one representative experiment.

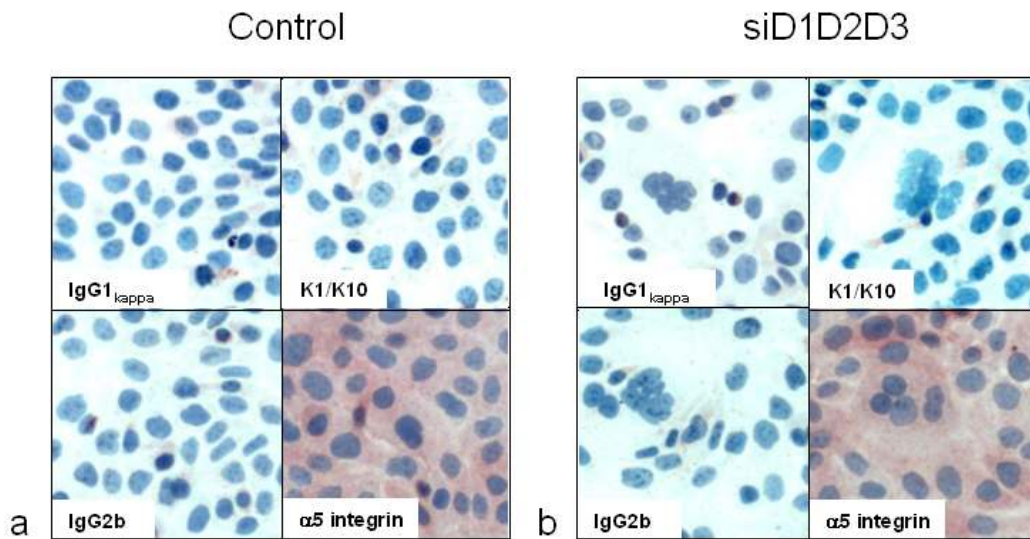
When measured by MTT assay, which indirectly indicates cell growth, the dual repressions of D-type cyclins caused growth suppressions by 35% in cyclin D1 and D2, 35.8% in cyclin D1 and D3 and 22% in cyclin D2 and D3 silenced cells, while repression of all the three D-type cyclins cooperatively suppressed HaCaT cell growth by 38% compared to the mock siRNA nucleofected controls in the 72 hour samples (Fig 9 first block of columns). In these cells 35% growth suppression was also detected after 96 hours, however, dual repression of D-type cyclins had no further effect on the cell growth after 72 hours, as compared to control transfections (Fig 9 second block of columns). At 168 hours after nucleofection, no difference in cell growth could be detected in any of the silenced cells (Fig 9 third block of columns).



**Fig. 9.** HaCaT cells with double and triple D-type cyclin silencing reveal decreased cell viability 72 hours after nucleofection. Contact inhibited and serum starved HaCaT keratinocytes were nucleofected with the combination of cyclin D1 D2, D1 D3, D2 D3 and D1 D2 D3 siRNAs and Control siRNA and were cultured for 72, 96 and 168 hours. Cell viability was measured by MTT assay. Data are indicated as absorbance values measured at 540 nm. Values shown are from two independent experiments (mean  $\pm$ SD).

***4.2.2 The expression of the proliferation related  $\alpha 5$  integrin and the differentiation related K1/K10 proteins shows no difference in combined D cyclin siRNA nucleofected cells compared to control cells.***

Previously it has been shown that  $\alpha 5$  integrin and K1/K10 are involved in the regulation of HaCaT cell proliferation and differentiation. A strong  $\alpha 5$  integrin expression appears in the highly proliferating, undifferentiated cells, while K1/K10 protein expression is highly elevated only in more differentiated, less proliferative cultures<sup>31</sup>. To further explore whether multiple silencing of D cyclins affects cell differentiation, we performed immunocytochemical stainings on cultured cells, to determine the expressions of proliferation ( $\alpha 5$  integrin) and differentiation (K1/K10) related proteins in HaCaT keratinocytes that were contact inhibited for one day and serum starved for five days. Cells were then harvested and nucleofected with cyclin D1, D2 and D3 siRNAs in suspension and were seeded onto cultured slides. Control cultures were treated similarly and transfected with mock siRNA for D-type cyclins. 72 hours after nucleofection we examined the protein expression of K1/K10 and  $\alpha 5$  integrin by immunocytochemistry. We detected no differences in  $\alpha 5$  or K1/K10 expressions in the triple silenced cells (Fig 10 b) compared to control cultures (Fig 10 a). A strong  $\alpha 5$  integrin staining was characteristic for both cultures, while K1/K10 expression was hardly detectable in the cells. The multinucleated cells did not differ from the normal cells in  $\alpha 5$  and K1/K10 expressions.



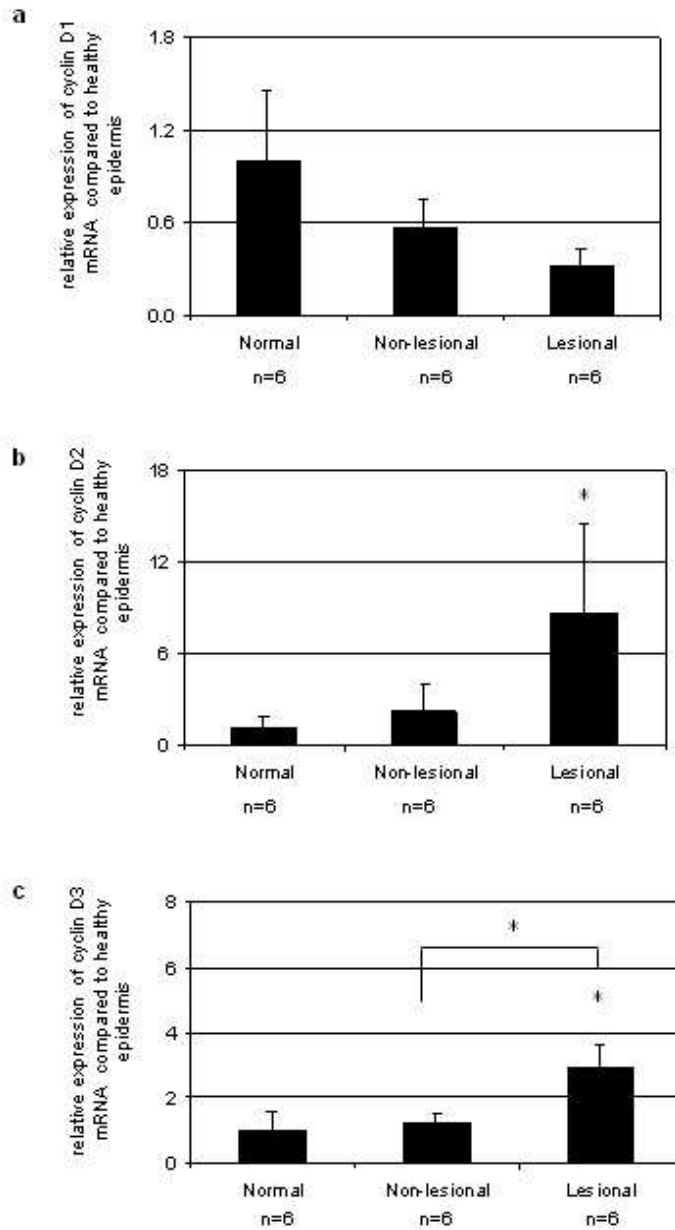
**Fig. 10.** Immunocytochemical detection of  $\alpha 5$  integrin and K1/K10 proteins in triple D-type cyclin silenced multinucleated HaCaT keratinocytes. Contact inhibited and serum starved HaCaT keratinocytes were nucleofected with the combination of cyclin D1, D2 and D3 siRNAs and Control siRNA and were cultured for 72 hours. K1/K10 protein expression was detectable neither in the triple silenced (b), nor in the control siRNA treated cells (a), while a strong cytoplasmic  $\alpha 5$  integrin staining was apparent both in the triple silenced (b) and in the Control siRNA treated cells (a), IgG1<sub>kappa</sub> is an isotype control staining for K1/K10 and IgG2b is control for  $\alpha 5$  integrin detection. Obj. 20x

### 4.3 Examination of D-type cyclins in psoriasis

#### 4.3.1 Cyclin D2 and D3, but not D1 mRNA expressions are significantly increased in psoriatic lesional epidermis, but the proportions of cyclin D2, D3 and D1 protein expressing cells are not changed

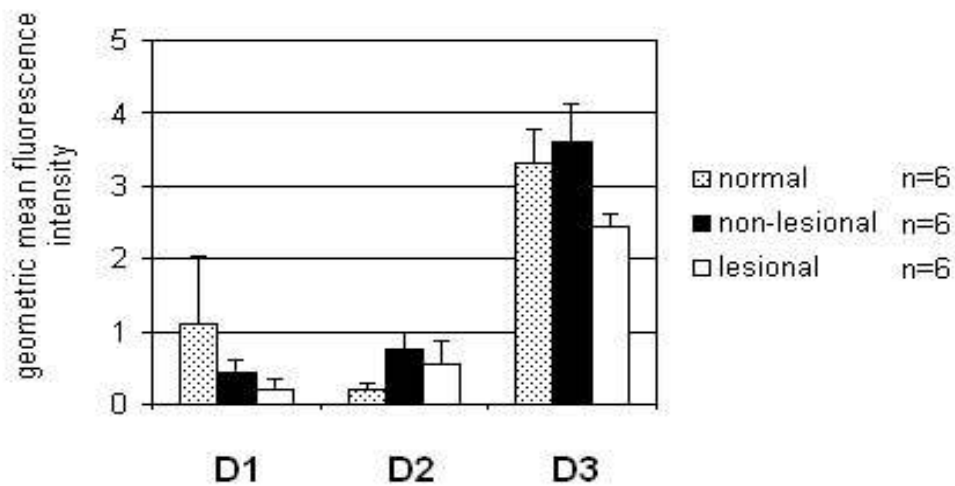
Because psoriasis is a skin disease characterized by abnormal proliferation of the normally quiescent basal keratinocytes, we aimed to compare the expression of cyclin D1, D2 and D3 in psoriatic lesional, non-lesional and healthy epidermis. We performed real-time RT-PCR and flow cytometric analysis on independent healthy (n=6), psoriatic uninvolved (n=6) and involved (n=6) epidermal samples. Results of the real-time RT-PCR analysis revealed no difference in cyclin D1 mRNA expression among normal, lesional and non-lesional epidermis (Fig 11 a). Cyclin D2 mRNA showed a significantly higher expression (8.61 fold) in lesional psoriatic epidermis compared to normal

epidermis (Fig 11 b). Although, a 3.94 fold increase was also detected between the psoriatic non-lesional and lesional epidermis in cyclin D2 mRNA expression, the difference here was not significant (Fig 11 b). Cyclin D3 mRNA expression was significantly higher in lesional epidermis compared to both non-lesional psoriatic (2.41 fold increase, Fig 11 c) and normal epidermis (2.92 fold increase Fig 11 c). Flow cytometry showed that among epidermal cells separated from normal, psoriatic lesional and non-lesional epidermis a low percentage of cells (2.28 +/- 0.8%, 6.83 +/- 3.69% and 0.25 +/- 0.04% of all cells) expressed cyclin D1, D2 type cyclins were expressed by similar very low percentage of cells (4 +/- 2.95%, 10.4 +/- 0.66% and 0.9 +/- 0.16% of all cells), while cyclin D3 protein was expressed by a larger percentage of cells (59.31 +/- 5.2%, 29.7 +/- 6.8% and 37.5 +/- 10% of all cells). We found no difference in cyclin D protein expression among normal, lesional and non-lesional psoriatic epidermis samples by flow cytometric analysis comparing the GeoMean results (Fig 12).



**Fig. 11.** Cyclin D1, D2 and D3 mRNA expression in psoriatic lesional, non-lesional and normal epidermis. The expression of cyclin D1 (a), D2 (b) and D3 (c) mRNAs were analyzed by real-time RT-PCR in healthy (n=6), psoriatic uninvolved (n=6) and involved (n=6) epidermal samples. Values are shown as relative expression compared to healthy epidermis (mean  $\pm$  SD, \* $p < 0.05$ , Student's t test).



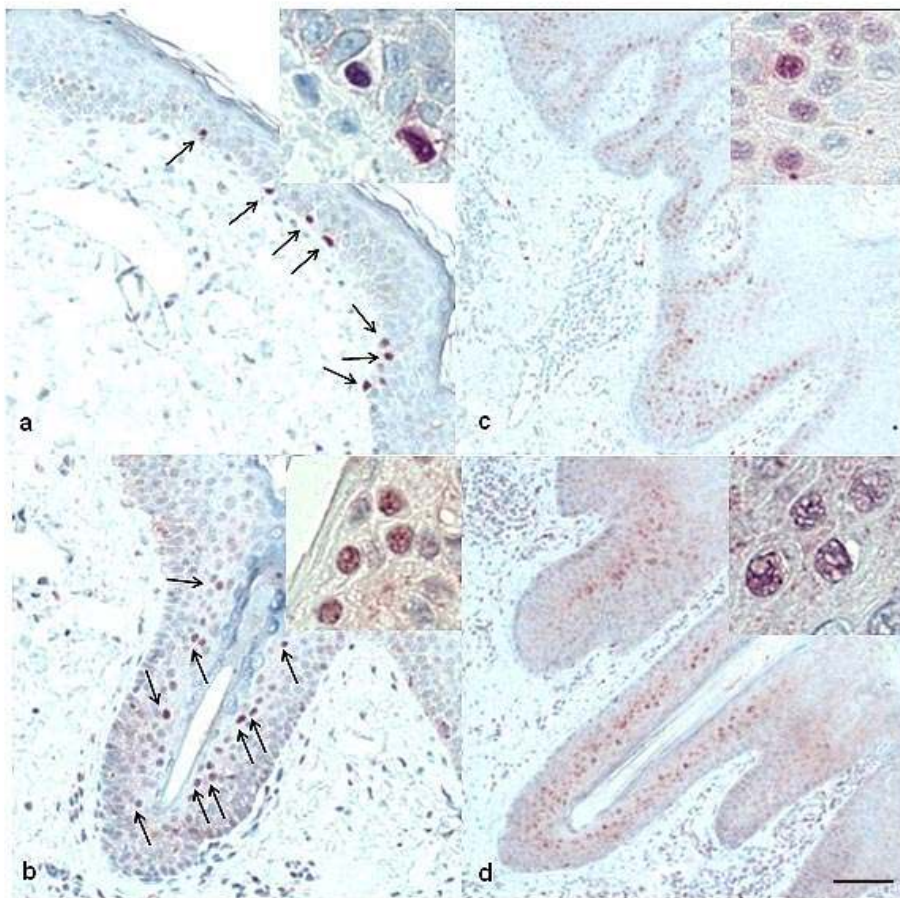


**Fig. 12.** Cyclin D1, D2 and D3 protein expression in psoriatic lesional and normal epidermis. The expression of cyclin D1, D2 and D3 proteins were analyzed by flow cytometry in healthy (n=6), psoriatic uninvolved (n=6) and involved (n=6) epidermal samples. The geometric mean fluorescence intensity (GeoMean) was calculated by the subtraction of the GeoMean of the cells stained with isotype-matched control immunoglobulin from the GeoMean of cells stained with antigen specific antibody, using CellQuest software. Values represent mean  $\pm$  SD.

***4.3.2 The absolute numbers of cyclin D1 expressing cells are increased in psoriatic lesional tissue, while the expression of cyclin D3 protein in lesional psoriatic epidermis appears similar to normal epidermis***

Although, we found no difference in cyclin D1 expression when we analyzed epidermal cell suspensions by flow cytometry, immunohistochemical staining showed increased numbers of cyclin D1 expressing keratinocytes in the basal layers of the lesional epidermis (Fig 13 c) compared to normal (Fig 13 a) and non-lesional (not shown) epidermis. In healthy epidermis, immunohistochemical detection showed distinct nuclear staining, but only rare isolated cells in the basal, immediately suprabasal cell compartments were positive for cyclin D1 (Fig 13 a arrows). Cyclin D3 positive staining, both nuclear and cytoplasmic, in normal epidermis could be detected only in the suprabasal layers of the hair follicles (Fig 13 b and d arrows), while in the interfollicular region it showed negative staining (not shown). In the lesional psoriatic

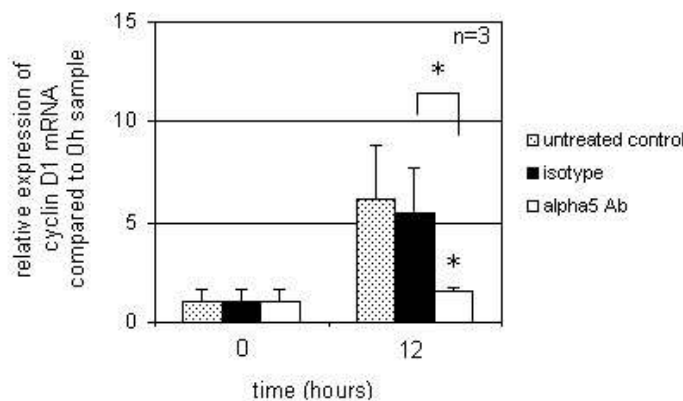
epidermis, many more cells in the basal and immediate suprabasal compartment showed nuclear as well as cytoplasmic staining for cyclin D1 (Fig 13 c). The proportion of cyclin D3 expressing cells showing nuclear and cytoplasmic staining were similar in lesional psoriatic skin compared to normal skin. We were not able to detect cyclin D2 expression by immunohistochemical analysis in any of the examined psoriatic and normal skin sections. This negative result was not due to technical constrains because with the same method using the same antibody we detected very clear positive stainings in skin tumors (data not shown).



**Fig. 13.** Immunohistochemical detection of cyclin D1 and D3 proteins in healthy and psoriatic lesional epidermis. Cyclin D1 positive cells are rare, and show distinct nuclear staining in the basal layer of normal epidermis (a). Intense cyclin D1 nuclear and a slight cytoplasmic staining can be detected in many more basal as well as suprabasal cells of the lesional psoriatic epidermis (c). Cyclin D3 staining is localized to the cytoplasm and nucleus, and detected only in the suprabasal cells of the hair follicles, both in normal (b) and in psoriatic lesional skin (d). Scale bar = 50  $\mu$ m.

### 4.3.3 Blocking of $\alpha 5$ integrin function results in a significant decrease of cyclin D1 mRNA expression in synchronized HaCaT keratinocytes

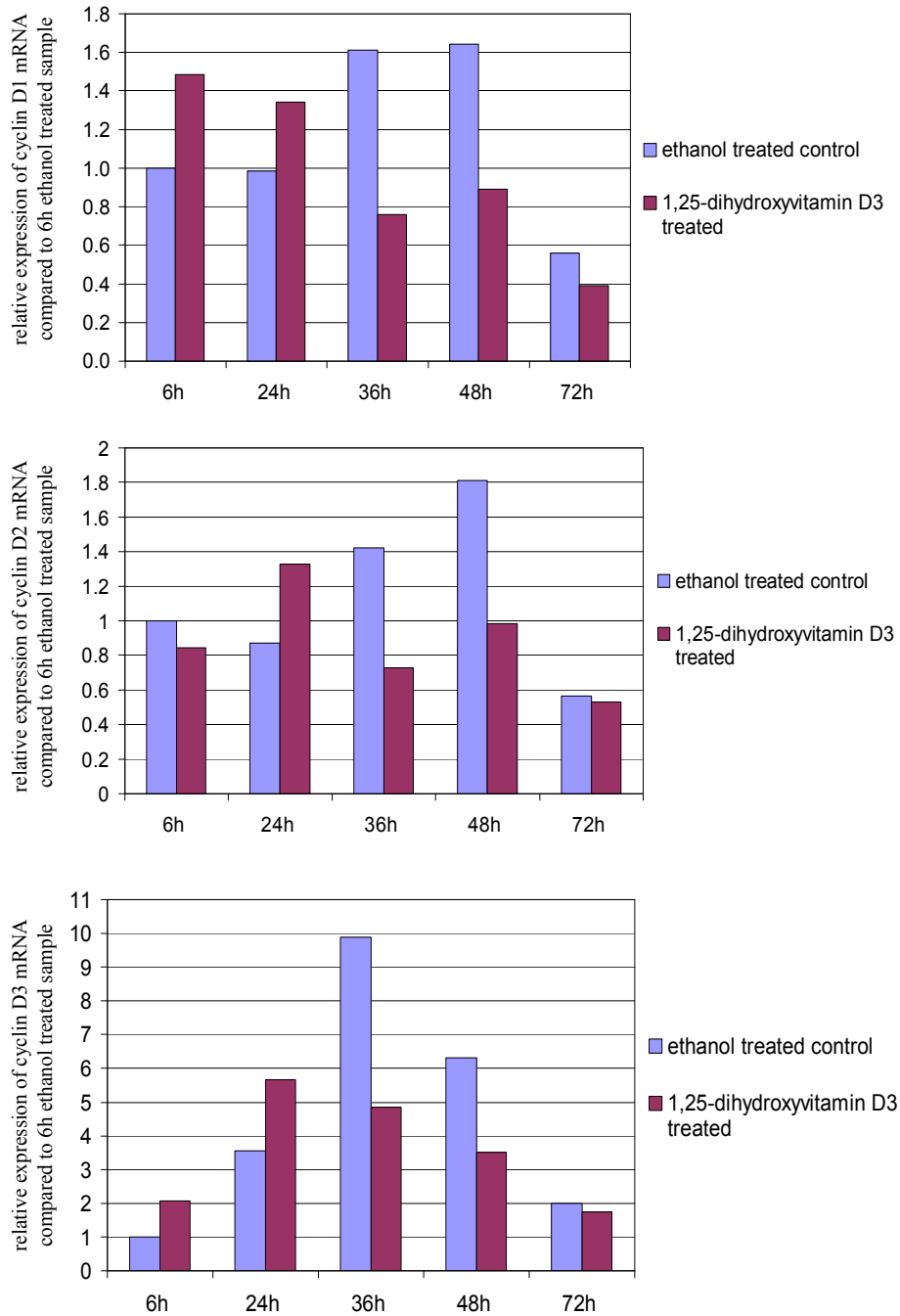
We were next interested in seeing whether  $\alpha 5$  integrin mediated signal transduction events could regulate the mRNA expression of cyclin D1 in the postquiescent HaCaT keratinocytes. HaCaT keratinocytes were synchronized as described and after harvest they were seeded onto culture plates. When cells were already attached and spread on the plates, about 5 hours after seeding, we added a purified mouse anti-human  $\alpha 5$  integrin neutralizing monoclonal antibody and an isotype control antibody to the cultures. Cells were then further cultured and 12 hours after release from cell quiescence cyclin D1 mRNAs were measured using real-time RT-PCR analyses (n=3). The mRNA expression of cyclin D1 was significantly inhibited in the specific antibody treated cultures compared both to the untreated control cells and the isotype matched immunoglobulin treated cells (Fig 14). The specific antibody treatment had no influence on the cyclin D2 and D3 expressions of the cells (data not shown).



**Fig. 14.** Blocking the function of  $\alpha 5$  integrin immediately after surface attachment in synchronized HaCaT keratinocytes leads to the down regulation of cyclin D1 mRNA expression. A neutralizing monoclonal antibody to human  $\alpha 5$  integrin was added to already attached, synchronized HaCaT keratinocytes 5 hours after release from cell quiescence and cells were further cultured. The effect of neutralizing antibody on cyclin D1 mRNA expression was detected by real-time RT PCR analysis. Values are indicated as relative expression compared to 0h untreated sample (n=3) (mean +/- SD, \*p < 0.05, Mann Whitney U test).

#### ***4.3.4 D-type cyclins could be possible targets of the antipsoriatic effect of 1,25-Dihydroxyvitamin D3***

We were next interested in seeing whether the antipsoriatic antiproliferative *1,25-Dihydroxyvitamin D3* could regulate the mRNA expressions of D-type cyclins in the postquiescent HaCaT keratinocytes. HaCaT keratinocytes were synchronized as described and after harvest they were seeded onto culture plates. When cells were already attached and spread on the plates, about 6 hours after seeding, we added *1,25-Dihydroxyvitamin D3* to the cultures. Cells were then further cultured and 24, 48 and 72 hours after release from cell quiescence cyclin D1, D2 and D3 mRNAs were measured using real-time RT-PCR analyses (n=2). The mRNA expression of all the three D-type cyclins were inhibited in the *1,25-Dihydroxyvitamin D3* treated cultures compared both to the untreated control cells and the vehicle (ethanol) treated cells (Fig 15).



**Fig. 15.** *1,25-Dihydroxyvitamin D3* treatment of synchronized HaCaT keratinocytes leads to the down regulation of all three D cyclin mRNAs. *1,25-Dihydroxyvitamin D3* was added to already attached, synchronized HaCaT keratinocytes 6 hours after release from cell quiescence and cells were further cultured. Cyclin D1, D2 and D3 mRNA expressions were detected by real-time RT PCR analysis. Values are indicated as relative expression compared to 24h sample (n=2) (mean +/- SD)

## Discussion

### *5.1 D-type cyclins: functional redundancy or unique role of the different isoforms in human keratinocyte proliferation and differentiation*

D-type cyclins are rate limiting factors of the G1 progression. Several reports suggest that D-type cyclins have non-overlapping functions in cell cycle regulation in specific cell types. Cyclin D1-mediated proliferation predominantly occurs in the immediate progeny of the stem-cell-rich bulge cells in the human hair follicle, indicating that cyclin D1 may be important for cells to exit the stem cell compartment<sup>44</sup> and cyclin D3 accumulates to high levels in the stratified squamous epithelial layers of postreplicative, differentiating cells but not in the superficial, terminally differentiated ones<sup>3</sup>. In normal human keratinocyte cultures, when quiescent (G0) K1/K10<sup>-</sup> keratinocytes traverse into the first cell cycle, they express cyclin D1, but not D2, as determined by antibody staining and flow cytometric analysis. As keratinocytes go into subsequent cell cycles in the in vitro culture, D1 protein expression disappears and cyclin D2 expression becomes clearly detectable. Corresponding to the lack of protein expression, cyclin D1 mRNA is not detectable 24 hours after plating, but it is clearly present as cells go into the first round of cell cycle (3-4 day cultures) and decreases in the subsequent cell cycles (Bata-Csorgo et al., 1996). In normal human skin a great number of keratinocytes express cyclin D1 in the basal and immediately suprabasal layer when they are induced to hyperproliferate 72 hours after a 2 MED UVB irradiation (data not shown).

We obtained similar results in immortalized keratinocytes (HaCaT), where the expression of cyclin D1 both at the mRNA and protein levels has increased in cells after leaving the G0 quiescent phase and cyclin D2 and D3 expression occurred in already cycling cells.

Although HaCaT keratinocytes showed differential expression in the synchronized culture, cyclin D1 gene-specific silencing had no effect on the proliferation and morphology of HaCaT keratinocytes during the interval between cellular quiescence and intense proliferation after release from quiescence. We did not detect a compensatory increase in silenced cells for the remaining D-type cyclin

mRNAs. It has been shown that the lack of cyclin D1 expression does result in significant reduction in mouse skin and mammary tumor development. However, complete elimination of tumor development was not observed in these models, suggesting that other cyclin/cdk complexes may partially compensate for the loss of cyclin D1 function<sup>33</sup>. It is also known that in knock-in mice expressing cyclin D2 in place of cyclin D1, cyclin D2 can replace cyclin D1's functions<sup>11</sup>. Our results also indicate that D2 and D3 cyclins could substitute for D1 in driving keratinocyte cell cycle.

Similar to D1 cyclin silencing, knocking down cyclin D2 and D3 alone has no effect on the proliferation and morphology of HaCaT keratinocytes. Silencing of the individual D-type cyclins did not result in a compensatory increase of the other two D-type cyclins in the cells. In mice, there is evidence that cyclin D2 and D3 can compensate for each other in driving the cell cycle of B lymphocytes<sup>24;26</sup>. In human bronchial epithelial (HBE) cells the independent targeting of D-type cyclins using small interfering RNAs, however results in cell growth suppression<sup>25</sup>. The fact that knocking down individual D cyclins had no effect in HaCaT cell proliferation and morphology in our experiments may be explained by the fact that these cells are not normal, but immortalized keratinocytes. The requirement for individual D-type cyclin functions in cell cycle regulation may vary among different cell types. There is evidence in mammalian embryonic fibroblasts that even normal cell cycles can take place without cyclin D CDK4/6 complex, in these cells cyclin E CDK2 alone is able to phosphorylate and inactivate pRb, activate E2F, and induce DNA synthesis<sup>23</sup>.

Contrary to the single knockdown experiments, double and triple silencing of D-type cyclins in HaCaT keratinocytes resulted in the formation of large multinucleated cells, S phase arrest and moderate growth suppression. Similar to the single cyclin silencing, no compensatory increase was detected in these cells for the remaining D-type cyclins. Interestingly, growth arrest and the formation of multinucleated cells were similar in all double silenced as well as in the triple silenced cultures. In HBE cells dual knockdown of D-type cyclins also resulted in growth suppression, however double or triple silencing of D-type cyclins had no effect on cell morphology<sup>25</sup>. Similar to our observation in HaCaT keratinocytes no compensatory overexpression of the remaining D-type cyclins was observed in HBE cells<sup>18</sup>. It has been also reported that inhibition of cyclin D3 expression by UV irradiation blocked HeLa and A2058 cells in G2 phase indicating that cyclin D3 activity was necessary for cell cycle progression through G2

phase into mitosis <sup>19</sup>. Small interfering RNA knockdown of cyclin D3 delayed the progression through G2 phase in HeLa cells, these cells failed to undergo cytokinesis correctly showing an aberrant mitotic phenotype characterized by lagging chromosomes that formed micronuclei after mitotic exit <sup>8</sup>. We have also investigated some molecular aspects of cell growth and differentiation in our multinucleated HaCaT cells. It has been shown that in continuously growing HaCaT keratinocytes the expression of  $\alpha 5$  integrin and K1/K10 was related to cell proliferation and differentiation <sup>31</sup>. Here we demonstrated that triple silenced multinucleated aberrant cells revealed no difference in K1/K10 and  $\alpha 5$  integrin expression compared to the control cells, suggesting that the morphological and proliferative changes, that occur due to triple silencing of D cyclins, did not influence the  $\alpha 5$  integrin pathway and did not affect K1/K10 expression.

These data indicate that in some cells the suppression or inhibition of a single D-type cyclin is sufficient to affect cell proliferation, while in others, such as our immortalized, hyperproliferative HaCaT cells, double or triple silencing of D-type cyclins must be applied for inducing cell proliferation and/or morphological changes. Interestingly, the combined D-type cyclin mRNA interference did not result in G0/G1 S block, but in G2/M block in the cells, as indicated by elevated S fractions in the cultures. Our observation is similar to the G2/M block that has been reported in HeLa cells by cyclin D3 silencing. In HeLa cells the aberrant mitosis was due to the failure in chromosome migration to the metaphase plate. These cells showed the same morphological characteristics (cells with multiple or micronuclei) as HaCaT cells in our experiments. In the cyclin D3 silenced HeLa cells lagging chromosomes were visualized which often decorated the astral side of the spindle microtubules. Protein expressions of the chromosomal passengers were a little lower or unaffected: survivin and borealin showed a slight decrease and the level of Aurora B was unchanged indicating, that these proteins were functioning normally <sup>8;19</sup>. While in HeLa cells an intact cyclin D3 function seems critical for normal G2/M progression, in HaCaT keratinocytes the lack of D3 function alone does not lead to G2/M block. The omission of at least two D-type cyclins was required for cell cycle disturbance in the G2/M phase. Interestingly we have not seen changes in G0/G1-S progression in our cells even when cells lacked all three D-type cyclins, indicating that similar to mammalian embryonic fibroblasts, normal G0/G1-S progress can take place without D-type cyclins in HaCaT



keratinocytes. In our experimental setup we have not been able to uncover the functional differences between the different D-type cyclins.

We conclude that the regulatory functions of D-type cyclins in keratinocytes are not completely redundant. Although knocking down cyclin D1, D2 and D3 alone in quiescent keratinocytes does not result in immediate changes in cell morphology and proliferation, the differential expression of these cyclins in cultured keratinocytes suggest that they may have other specific functions beside driving the cell cycle. Furthermore, D-type cyclins do not seem essential for G0/G1 S progression in HaCaT keratinocytes, but at least two of them have to function for normal G2/M transition.

### *5.2 D-type cyclins: participants in the pathogenesis of psoriasis and new therapeutical targets*

Although not yet characterized, beside cell cycle regulation D-type cyclins have multiple functions, as indicated by increasing data. The fact that different cell types differentially express D-type cyclins also supports this hypothesis. Cyclin D3 is ubiquitously expressed in seventy different human cultured cell types while D1 and D2 is not. Complementary analyses of human and mouse tissues at different stages of foetal and postnatal development revealed a correlation between cyclin D3 abundance and mature-differentiated phenotypes, indicating a possible role for cyclin D3 in the induction and maintenance of terminal differentiation<sup>3</sup>. In previous work using the same synchronized HaCaT keratinocyte model we found that the highly proliferating, cyclin D1 expressing, immediately postquiescent cells downregulate the early differentiation markers K1/10 at the mRNA levels<sup>31</sup>. Overexpressed cyclin D1 in HaCaT keratinocytes results in deregulation of tissue architecture with altered localization and impaired differentiation, these cells lack terminal differentiation and exhibit a more autonomous growth, even form keratoacanthoma-like tumors in vivo<sup>9</sup>.

HaCaT cells, although immortalized and genetically abnormal, are considered to be a good model for human keratinocytes. With their high proliferative potential, they especially resemble keratinocytes found in the skin disease psoriasis. Psoriasis can be characterized with an excessive basal cell proliferation and perturbed keratinocyte differentiation, which are regarded as central pathologic features of the disease. Because hyperproliferation in the psoriatic epidermis is due to activation of the normally quiescent cells in the  $\beta 1$  integrin<sup>+</sup> K1/K10<sup>-</sup> basal cell compartment<sup>6</sup>, we expected the expression of D1 type cyclin to be increased both at the mRNA and protein levels in

psoriatic epidermis. Contrary to our expectation, we detected a significant increase in cyclin D2 and D3 mRNA expression in chronic plaques of lesional psoriatic epidermis compared to normal epidermis, but the expression of cyclin D1 mRNA showed no difference. By flow cytometric analysis, the percentage of cells expressing D1, D2 and D3 cyclins was similar in the psoriatic epidermis as in the normal and the non-lesional epidermis. The percentage of D1 and D2 protein expressing cells was low relative to the percentage of D3 protein expressing cells in all samples. This result is similar to our previous observation showing that the increased number of cycling keratinocytes in the psoriatic  $\beta 1$  integrin<sup>+</sup> K1/K10<sup>-</sup> basal compartment did not result in an overall increase in the size of this cell compartment, indicating that the proportion of  $\beta 1$  integrin<sup>+</sup> K1/K10<sup>-</sup> cells that self-renew (to  $\beta 1$  integrin K1/K10<sup>-</sup> cells) is the same in psoriatic epidermis as it is in normal epidermis, and that excess cells are committed toward differentiation<sup>6</sup>.

Immunostaining of tissue sections revealed that the psoriatic lesional epidermis contained more cells in absolute numbers that showed distinct nuclear and some cytoplasmic expression of cyclin D1 protein than the normal and psoriatic non-lesional epidermis where we could detect only nuclear staining in a few basal layer cells. Xu et al. found that cyclin D1, D2 and D3 were not detectable by immunohistochemistry in the rapidly proliferating hair-producing cells of the anagen bulb while these cells were strongly positive for Ki-67 and retinoblastoma protein<sup>44</sup>. Contrary to these data, we found that cyclin D3 protein was expressed in the cytoplasm and nucleus of the suprabasal cells in the hair follicles and the proportion of positively stained cells seemed to be similar in the psoriatic lesional, non-lesional and normal epidermis. We used different antibodies for flow cytometry and immunohistochemistry because neither of the antibodies were suitable for both methods. We could not detect cyclin D2 in normal and lesional psoriatic epidermis by immunohistochemistry although some skin tumors (squamous cell carcinoma and melanoma) that we stained with the same antibody showed clear positive immunostaining of cyclin D2 (data not shown).

Beside transcriptional and posttranscriptional regulation, the expressions of D-type cyclins are regulated by targeted degradation via the ubiquitine pathway<sup>13 16 46 29</sup>. The fact that there are more cyclin D1 positive cells in the stable psoriatic lesion localized both in the basal as well as in the immediate suprabasal compartment of the epidermis, at the same time no increase in D1 mRNA can be detected in the psoriatic samples, indicate a possible dysfunction in the degradation of cyclin D1 in this disease,

that could be partially responsible for keratinocyte hyperproliferation and abnormal differentiation of the basal keratinocytes. It is possible that by retaining cyclin D1 expression, the highly proliferative  $\beta 1$  integrin<sup>+</sup> K1/K10<sup>-</sup> basal compartment keratinocytes in the psoriatic epidermis protect their stem cell potential. Indeed, primary cultured keratinocytes with induced cyclin D1 transgene expression show resistance to calcium-induced terminal differentiation and continue to replicate in vitro<sup>45</sup>. The significant increase in the relative expressions of cyclin D2 and D3 mRNAs in the psoriatic lesional epidermis with no change in protein expressions indicates posttranscriptional dysregulation of these cyclins in psoriatic keratinocytes.

It is well documented that in the skin, as in other tissues, keratinocyte integrins are important regulatory molecules in epidermal homeostasis. The main receptor of fibronectin,  $\alpha 5$  integrin is abnormally overexpressed in uninvolved keratinocytes of the in vivo psoriatic epidermis<sup>5</sup> and the A domain of fibronectin (EDA<sup>+</sup>) is present at the basement membrane zone of uninvolved psoriatic skin while absent in normal control skin<sup>38</sup>. Upon in vitro activation there is enhanced EDA<sup>+</sup> fibronectin expression in psoriatic uninvolved keratinocytes compared to normal cells<sup>37</sup>. The finding that  $\alpha 5$  integrin regulates cyclin D1 mRNA expression in quiescent HaCaT keratinocytes indicate that the abnormally active fibronectin- $\alpha 5$  integrin ligand-receptor induced pathway in the psoriatic non-lesional keratinocytes could contribute to keratinocyte hyperproliferation in psoriasis. That cyclin D1 expression is dependent on intact  $\alpha 5$  integrin function has also been indicated in the keratinocyte derived SCC12 cells, in which binding of the ganglioside GT1b to  $\alpha 5$  integrin inhibited the expression of cyclin D1<sup>40</sup>.

We have also found that in our HaCaT keratinocyte model system the antipsoriatic 1,25-Dihydroxyvitamin D3 had an effect on all D-type cyclin mRNA expression (data not shown), indicating that D-type cyclins could be the target of the 1,25-Dihydroxyvitamin D3 therapeutic effect in psoriasis.

## 6. Summary

D-type cyclins are regarded as essential links between cell environment and the core cell cycle machinery. In our study we intended to gain data on the normal and abnormal functions of D-type cyclins including cell cycle regulation and pathogenesis of psoriasis. In this study we show that the G<sub>0</sub>-G<sub>1</sub>/S phase of HaCaT keratinocyte cell cycle is characterized by D1-type cyclin expression, while during the repeated rapid turnover of highly proliferating cells, the expression of cyclin D2 and D3 dominates. Knocking down cyclin D1, D2 or D3 mRNA alone resulted in no change of cell proliferation and morphology, while those cells that were nucleofected with two or three D cyclin siRNAs at the same time, formed large multinucleated cells after silencing. The combined silencing of D-type cyclins resulted in S phase arrest and moderate growth suppression. Our data have not revealed the specific functions for different D-type cyclins, rather it showed that in cell cycle regulation these cyclins have functional redundancy. Furthermore, D-type cyclins do not seem essential for G<sub>0</sub>/G<sub>1</sub> S progression in HaCaT keratinocytes, but at least two of them have to function for normal G<sub>2</sub>/M transition. Next we tried to determine the role of D-type cyclins in the pathogenesis of psoriasis. We have found that increased numbers of cyclin D1 expressing keratinocytes were located in the basal layers of the lesional psoriatic epidermis compared to both normal and non-lesional epidermis without increased expression of cyclin D1 mRNA, suggesting a possible dysfunction in the degradation of cyclin D1 protein. We also detected a significant increase in D2 and D3 cyclin mRNA expression in psoriatic epidermis compared to normal epidermis with no difference in protein expressions. These data suggest a possible role for D-type cyclins in the excessive basal cell proliferation and perturbed keratinocyte differentiation that occurs in the psoriatic epidermis. We have also shown that blocking  $\alpha$ 5 integrin function by a neutralizing antibody in HaCaT keratinocytes down-regulated the expression of D1 cyclin mRNA without affecting the expressions of cyclin D2 and D3 indicating a regulatory role for  $\alpha$ 5 integrin in the expression of D1 cyclin. In our HaCaT keratinocyte model system the antipsoriatic 1,25-Dihydroxyvitamin D<sub>3</sub> had an effect on all D-type cyclin mRNA expression, indicating that D-type cyclins could be the target of therapies in psoriasis.

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