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

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Summary of Ph.D. thesis

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1. General 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 phosphorilation 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. Three D-cyclins - cyclin D1, D2 and D3 - operate in mammalian cells and play a major role in the positive regulation of G1 progression. Enforced overexpression of D type cyclins can shorten the G1 interval in cultured cells. Following mitogen stimulation of quiescent cells, genes encoding D type cyclins get activated at the beginning of the G1 phase, and then the produced D type cyclins bind to Cdk4 or Cdk6 to activate their kinase activity. Microinjected antibodies or antisense constructs directed against D type cyclins can interfere with G1 phase progression, without affecting other cell cycle intervals.

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. 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. 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.

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

Quiescent (G0 phase) basal (keratin1/keratin10 negative) K1/K10- 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. 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. 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.

The HaCaT keratinocyte cell line is a spontaneously transformed human epithelial cell line derived from a histologically normal skin specimen. 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 posess the ability to form a normally differentiated epidermis-like epithelium under appropriate conditions. 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.

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. In HaCaT epithelia as in the epidermis in situ proliferation is restricted to the basal layer. In the cyclin D1 overexpressed HaCaT cell-organotypic co-culture BrdU-positive nuclei were only seen in the basal layer.

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, cyclin D2 plays a role in macrophage activation and spermatogonial differentiation, while cyclin D3 has a role in the promotion and maintenance of the terminally differentiated state of some cell types.

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. 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.

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. 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.

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. 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. 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. 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.

1.3 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. 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. 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.

2. Aims

- to characterize the expression pattern of D-type cyclins in synchronized HaCaT keratinocytes.
- to investigate the funtions 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 α5 integrin regulates the expression of cyclin D1
- to assess the effect of D3 vitamin on the expression of D-type cyclins

3. Methods

3.1 Cell cultures

HaCaT cells, the spontaneously immortalized non-tumorigenic human keratinocyte-derived cell line, (kindly provided by Dr N.E. Fusening, Heidelberg, Germany) were cultured in a high-glucose Dulbecco's modified Eagle' medium supplemented with 10% fetal bovine serum, L-glutamine, antibiotics (penicillin and streptomycin) and an antimycotic (amphotericin B).

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 (Pivarcsi et al., 2001). Samples were collected 0, 12, 24, 36, 48, 72, 96 and 168h following the end of the synchronization process.

3.4 Quantitative reverse transcription (RT)-PCR

For quantitative real time RT-PCR, 1 μg purified total RNA was reverse transcribed with the iScript cDNA Synthesis Kit according to the manufacturer's instructions. After RT, amplification reactions were carried out with an iCycler machine using the appropriate primer pairs and probes. To detect 18S rRNA (as endogenous control) we used sequence-specific TaqMan probes. The amount of cDNA was calculated on the basis of the threshold cycle value, and was standardized by the amount of housekeeping gene (18S rRNA).

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 isothyonate (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 (Beckton Dickinson). Results were expressed either as the geometric mean fluorescence intensity (GeoMean) calculated by substraction (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 NucleofectorTM 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^{TM} 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 x 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 (Sonkoly et al., 2005).

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

1x10^6 synchronized HaCaT keratinocytes were seeded into 25 cm^2 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 x 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 Apo2.7 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 x 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 FacsCalibur 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 when 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 substraction 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 and Discussion

4.1 D type cyclins: functional redundancy or unique role of the different isotypes 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. In normal human keratinocyte cultures, when quiescent (G0) K1/K10$^-$ 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 with 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. 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.

We obtained similar results in immortalized keratinocytes (HaCaT) both at the mRNA and protein level. The highest level of cyclin D1 expression was detected 24 hours after release from cell quiescence when cells started to proliferate. Cyclin D2 and D3 also showed low levels of expressions in quiescent cells (0 hours). Cyclin D2 and D3 mRNA and protein levels gradually increased in the cells after release from cell quiescence, but their peak expression occurred at 48-72 hours in already proliferating cells. Both cyclin D2 and cyclin D3 expressions showed maximum levels at 48 and 72 hours. As cell cultures became confluent mRNA levels for all cyclins have decreased.

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. 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. 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 had 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. In human bronchial epithelial (HBE) cells the independent targeting of D-type cyclins using small interfering RNAs, however results in cell growth suppression. 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.

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. Similar to our observation in HaCaT keratinocytes no compensatory overexpression of the remaining D-type cyclins was observed in HBE cells. 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. 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. We have also investigated some molecular aspects of cell growth and differentiation in our multinucleated HaCaT cells. It has been shown that in continously growing HaCaT keratinocytes the expression of α5 integrin and K1/K10 was related to cell proliferation and differentiation. Here we demonstrated that triple silenced multinucleated aberrant cells revealed no difference in K1/K10 and α5 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 α5 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. 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.

4.2 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 are 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. 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. 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.

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^{+} K1/K10^{-} basal cell compartment, 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 β1 integrin$^+$ K1/K10$^-$ basal compartment did not result in an overall increase in the size of this cell compartment, indicating that the proportion of β1 integrin$^+$ K1/K10$^-$ cells that self-renew (to β1 integrin K1/K10$^-$ cells) is the same in psoriatic epidermis as it is in normal epidermis, and that excess cells are committed toward differentiation.

Immunostainig 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. 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.

Beside transcriptional and posttranscriptional regulation, the expressions of D- type cyclins are regulated by targeted degradation via the ubiquitine pathway. 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 β1 integrin$^+$ K1/K10$^-$ 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. 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, α5 integrin is abnormally overexpressed in uninvolved keratinocytes of the in vivo psoriatic epidermis and the A domain of fibronectin (EDA$^+$) is present at the basement membrane zone of uninvolved psoriatic skin while absent in normal control skin. Upon in vitro activation there is enhanced EDA$^+$ fibronectin expression in psoriatic uninvolved keratinocytes compared to normal cells. The finding that α5 integrin regulates cyclin D1 mRNA expression in quiescent HaCaT keratinocytes indicate that the abnormally active fibronectin-α5 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 α5 integrin function has also been indicated in the keratinocyte derived SCC12 cells, in which binding of the ganglioside GT1b to α5 integrin inhibited the expression of cyclin D1.

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, indicating

that D-type cyclins could be the target of the 1,25-Dihydroxyvitamin D3 therapeutic effect in psoriasis.

5. Summary

- We have demonstrated that the G0-G1/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.
- We have showen that in HaCaT keratinocytes knocking down cyclin D1, D2 or D3 mRNA alone results 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 leading to S phase arrest and moderate growth suppression.
- By using immunohistochemistry we have shown that increased numbers of cyclin D1 expressing keratinocytes are located in the basal layers of the lesional psoriatic epidermis compared to both normal and non-lesional epidermis.
- We have 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.
- We have demonstrated 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.
- We revealed that the antipsoriatic 1,25-Dihydroxyvitamin D3 had an effect on all D type cyclin mRNA expression in HaCaT keratinocytes.

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