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ORIGINAL ARTICLE

# Differential Expression of D-Type Cyclins in HaCaT Keratinocytes and in Psoriasis

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In this study, we show that the $G_0$–$G_1$/S phase of HaCaT keratinocyte cell cycle is characterized by D1-type cyclin expression, whereas during the repeated rapid turnover of highly proliferating cells, the expression of cyclins D2 and D3 dominates. Knocking down cyclin D1 mRNA resulted in no change of cell proliferation and morphology, indicating that D2 and D3 cyclins could substitute for D1 in driving the cell cycle. Increased numbers of cyclin D1-expressing keratinocytes were found 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 cyclin D2 and D3 mRNA expressions in psoriatic epidermis compared to normal epidermis with no difference in protein expressions. Blocking $\alpha$5-integrin function by a neutralizing antibody in HaCaT keratinocytes downregulated the expression of cyclin D1 mRNA without affecting the expressions of cyclin D2 and D3 indicating a regulatory role for $\alpha$5-integrin in the expression of cyclin D1. Our data suggest a possible role for D-type cyclins in the excessive basal-cell proliferation and perturbed keratinocyte differentiation in the psoriatic epidermis.

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## INTRODUCTION

D-type cyclins are one of the key components of the cell-cycle machinery (Sherr and Roberts, 2004). Three D cyclins, cyclins D1, D2, and D3, operate in mammalian cells and play a major role in positive regulation of G1 progression (Ciemerych *et al.*, 2002). In the developing skin of mice, cyclin D1 is present in keratinocytes and is absent from developing hair follicles, whereas cyclin D2 exhibits an opposite pattern of expression (Aguzzi *et al.*, 1996). 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 (Bartkova *et al.*, 1998).

Quiescent ($G_0$ phase) basal K1/K10$^-$ (keratin1/keratin10-negative) keratinocytes freshly isolated from human skin express, D1- but not D2-type cyclin when they almost synchronously traverse from $G_0$–$G_1$ 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 cyclin D2 becomes clearly detectable. The differential expression of cyclins D1 and D2 in keratinocytes suggests specific functions for D-type cyclins and provides direct evidence that the $G_0$–$G_1$–S progression of quiescent keratinocytes is distinct from the $G_1$–S traverse of already cycling cells (Bata-Csorgo *et al.*, 1996). 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 (Xu *et al.*, 2003). In contrast, cyclin D3 was reported to accumulate to high levels in the stratified squamous epithelial layers of postreplicative, differentiating cells in mice (Bartkova *et al.*, 1998). To understand better cyclin D functions in keratinocytes, we followed their expression in synchronized HaCaT keratinocyte cultures. To determine whether cyclin D1 function was essential for quiescent HaCaT keratinocytes to proliferate after release from contact inhibition and serum starvation, we knocked down the expression of cyclin D1 mRNA in synchronized HaCaT keratinocytes by RNA interference.

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 (Bata-Csorgo *et al.*, 1993). 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

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*Abbreviations: K1/K10, keratin1/keratin10; RT, reverse transcription*

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entry among uninvolved but not normal keratinocytes (Bata-Csorgo *et al.*, 1998). Concordantly, α5-integrin the receptor of fibronectin, but not α2 or α3, is overexpressed in the *in vivo* non-lesional psoriatic epidermis (Pellegrini *et al.*, 1992; Bata-Csorgo *et al.*, 1998). The role of abnormal α5-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 (Carroll *et al.*, 1995). 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 α5-integrin in the regulation of cyclin D1 mRNA expression.

## RESULTS

### The $G_0$–$G_1$/S phase of HaCaT keratinocyte cell cycle is characterized by the appearance of D1-type cyclin expression, whereas during the repeated rapid turnover of highly proliferating cells expression of cyclins D2 and D3 dominates

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 shown previously by cell-cycle analysis of PI-stained cells (Pivarcsi *et al.*, 2001). 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 hours after release from cell quiescence, closely followed by increasing number of cells entering S phase. Cyclin D1 expression is preceded by the increase of α5-integrin mRNA in the cells; at the same time mRNAs for the early differentiation markers K1/K10 substantially decrease. By the end of a 1-week culture period, keratin expression reoccurs in the cells parallel to suppression of α5-integrin and cyclin D1 expression and with reduction of proliferation (Pivarcsi *et al.*, 2001). The expression of cyclins 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 after the end of the synchronization process, using real-time reverse transcription PCR (RT-PCR). The serum-starved, contact-inhibited HaCaT keratinocytes, similar to our previous observation, expressed a very low level of cyclin D1 mRNA (0 hour sample), which increased significantly with time after passaging and serum re-addition. The highest level of cyclin D1 mRNA expression (10.7-fold increase compared to 0 hour samples) was detected 24 hours after release from cell quiescence when cells started to proliferate (Figure 1a). Cyclin D2 and D3 mRNAs also showed low levels of expression in quiescent cells (0 hour). 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 (Figures 1b and c). Both cyclin D2 (Figure 1b) and cyclin D3 (Figure 1c) 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 0 hour samples). As

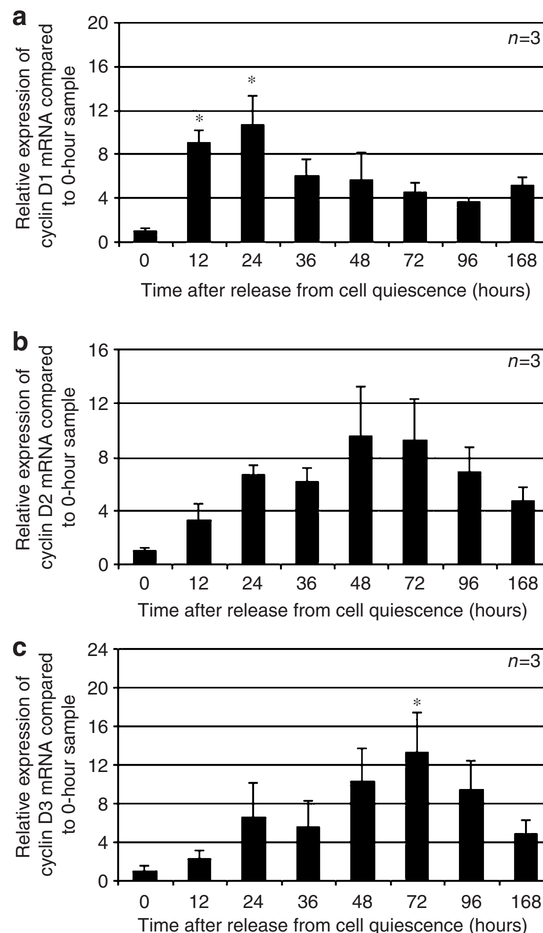


**Figure 1. Cyclin D1, D2, and D3 mRNA expressions in synchronized HaCaT keratinocytes.** HaCaT keratinocytes ($n=3$) were arrested by serum deprivation and contact inhibition, then stimulated to re-enter the cell cycle by passaging them into serum-containing medium. Changes in the expression of cyclin D1, D2, and D3 mRNAs were analyzed by real-time RT-PCR (**a–c**) at the times indicated after release from cell quiescence. Values are shown as relative expression compared to 0 hour sample (mean±SD, *$P<0.05$, Dunnett's test).

cell cultures became confluent mRNA levels for all cyclins have decreased.

The levels of cyclin D1, D2, and D3 protein expressions showed similar kinetics compared 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) (Figure 2a), 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) (Figure 2c). Both cyclin D1 and D2 expressions increased considerably after release from cell quiescence (Figures 2a and b). The highest level of cyclin D1 protein expression (2.82-fold increase in geometric mean fluorescence intensity/GeoMean/compared to the 0 hour samples) was detected 24 hours after release from cell quiescence (28.97±3.3% cells were positive for cyclin D1) (Figure 2a). Cyclin D2 expression peaked later at 72 hours in the already highly proliferative cultures (5.17-fold increase in GeoMean at 72 hours compared to 0 hour samples, Figure 2b), where 83.41±3.6% of the cells showed

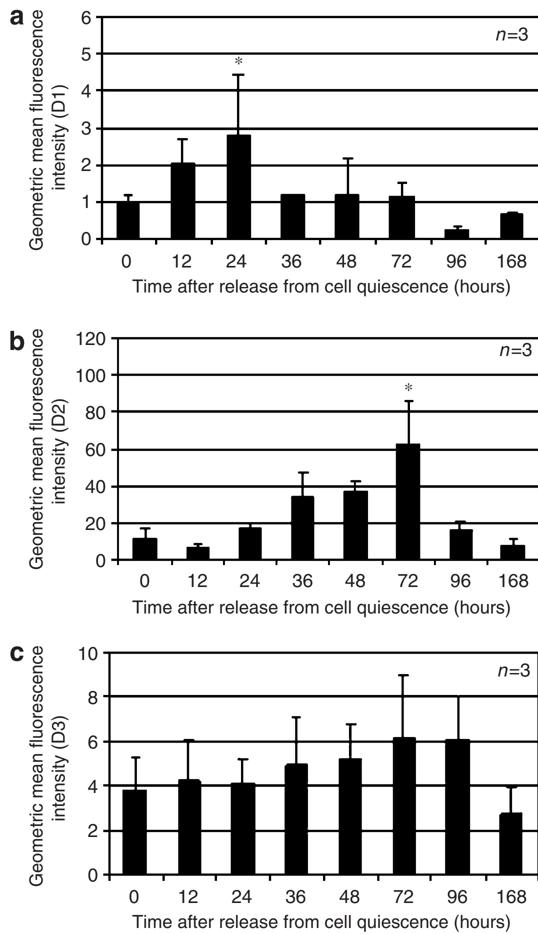


**Figure 2. Cyclin D1, D2, and D3 protein expressions in synchronized HaCaT keratinocytes.** HaCaT keratinocytes ($n=3$) were synchronized by contact inhibition and serum starvation (0 hour), and then released from cell quiescence by serum re-addition and passage. Flow cytometric analysis was used to detect the expression of (**a**), cyclin D1, (**b**) D2, and (**c**) D3 proteins 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).

positive staining. For D3 protein expression only a slight increase was detected in the cells at 72 and 96 hours compared to the 0 hour level (1.6- and 1.57- fold increase in GeoMean); however, 66.08 ± 6.67% cells were positive for cyclin D3) (Figure 2c).

### Neither cell morphology nor proliferation, is affected by cyclin D1 gene-specific silencing in HaCaT keratinocytes released from cell quiescence

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 5 days. Cells were then harvested and nucleofected with cyclin D1 small-interfering RNA (siRNA) in suspensions and then plated and cultured. Two independent experiments were carried out, and 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 downregulated cyclin D1 expression in the transfected HaCaT keratinocytes at 24, 48, and 72 hours after the plating of the cells (Figure 3a). Silencing of D1 mRNA expression had no effect on the D2 and D3 mRNA expressions (Figures 3b and c). Cell numbers determined by thiazolyl blue tetrazolium bromide assay at the same time points were similar in all cultures (Figure 3d). Cell and colony morphology in D1 cyclin silenced HaCaT cultures did not show any obvious difference compared to both controls (Figure 4).

### Cyclin D2 and D3 but not cyclin D1 mRNA expressions are significantly increased in psoriatic lesional epidermis, but the proportions of cyclins 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 cyclins 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 (Figure 5a). Cyclin D2 mRNA showed a significantly higher expression (8.61-fold) in lesional psoriatic epidermis compared to normal epidermis (Figure 5b). 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 (Figure 5b). Cyclin D3 mRNA expression was significantly higher in lesional epidermis compared to both non-lesional psoriatic (2.41-fold increase; Figure 5c) and normal epidermis (2.92-fold increase; Figure 5c).

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 a similar very low percentage of cells (4 ± 2.95, 10.4 ± 0.66, and 0.9 ± 0.16% of all cells), whereas 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 (Figure 6).

### The absolute numbers of cyclin D1 expressing cells are increased in psoriatic lesional tissue, whereas 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

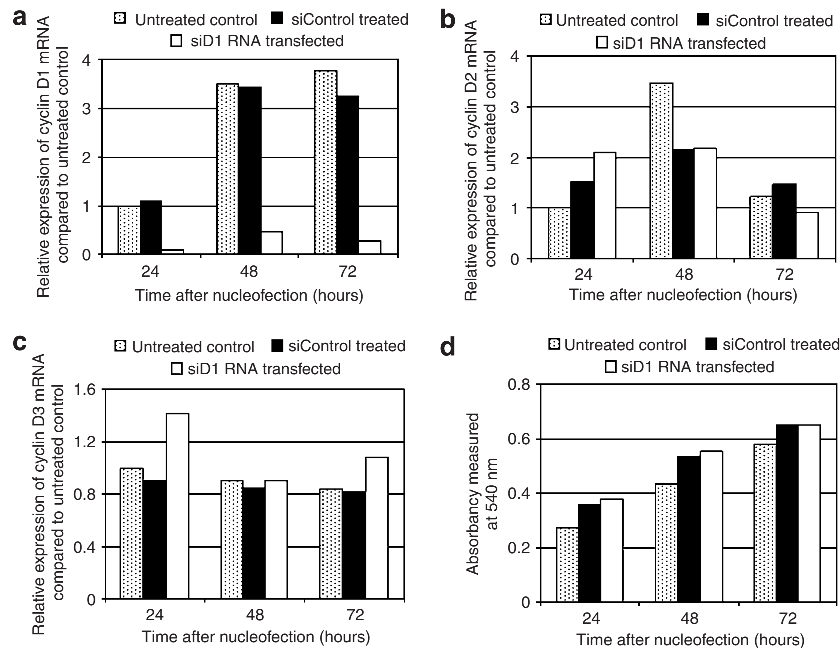


**Figure 3. Gene-specific silencing by siRNA effectively downregulates the expression of cyclin D1 and does not affect cell viability of synchronized HaCaT keratinocytes.** Contact-inhibited and serum-starved cyclin D1 siRNA, control siRNA-transfected and -untransfected HaCaT keratinocytes were cultured for 24, 48, and 72 hours. (**a**) Cyclin D1, (**b**) D2, and (**c**) D3 mRNA expressions were determined by real-time RT-PCR. Data are indicated as fold expressions compared to the 24 hours untransfected sample. Values shown are from a representative experiment. Cell viability was measured by thiazolyl blue tetrazolium bromide assay. Data are indicated as absorbance values measured at 540 nm. (**d**) Values shown are from the same representative experiment.

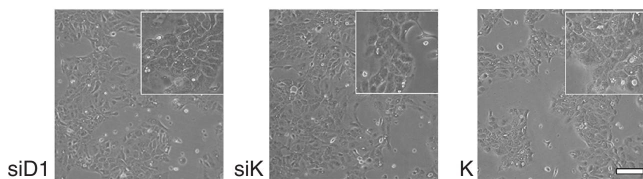


**Figure 4. Gene-specific silencing by cyclin D1 siRNA does not alter the morphology of HaCaT keratinocytes.** Contact-inhibited and serum-starved cyclin D1 siRNA, control siRNA-transfected and -untransfected HaCaT keratinocytes were cultured for 72 hours after passaging. Cell morphology was detected by a Nikon Eclipse TS100 microscope and photographed by a Nikon Coolpix 4500 digital camera. Bar = 50 $\mu$m.

cytometry, immunohistochemical staining showed increased numbers of cyclin D1 expressing keratinocytes in the basal layers of the lesional epidermis (Figure 7c) compared to normal (Figure 7a) and non-lesional (data 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 (Figure 7a arrows). Cyclin D3-positive staining, both nuclear and cytoplasmic, in normal epidermis could be detected only in the suprabasal layers of the hair follicles (Figures 7b and d arrows), whereas it showed negative staining in the interfollicular region (data 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 (Figure 7c). The proportion of cyclin D3 expressing cells showing nuclear and cytoplasmic staining was 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 constraints, because with the same method using the same antibody we detected very clear positive stainings in skin tumors (data not shown).

### Blocking of α5-integrin function results in a significant decrease of cyclin D1 mRNA expression in synchronized HaCaT keratinocytes

We were next interested in seeing whether α5-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 α5-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 (Figure 8). The specific antibody treatment had no influence on the cyclin D2 and D3 expressions of the cells (data not shown).

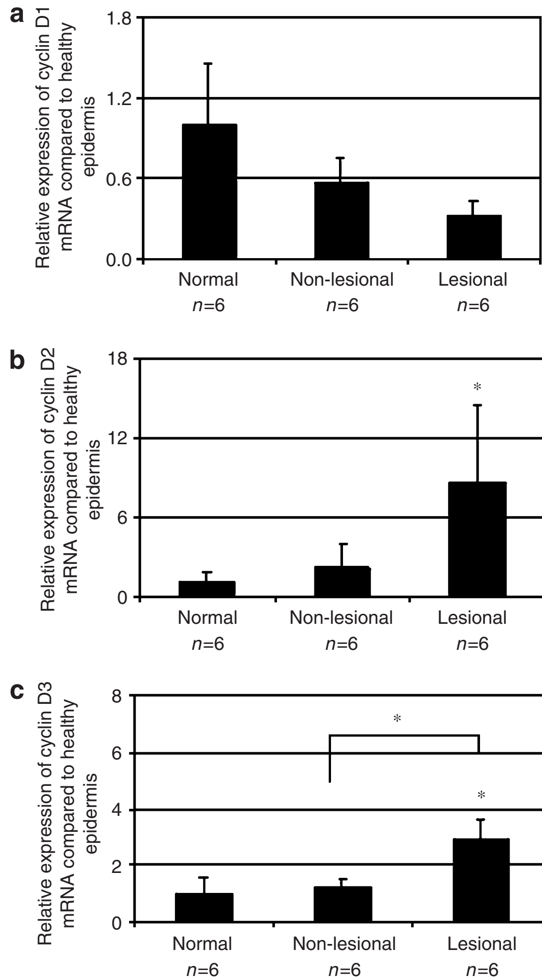


**Figure 5. Cyclin D1, D2, and D3 mRNA expressions in psoriatic lesional, non-lesional, and normal epidermis.** The expression of (**a**) cyclin D1, (**b**) D2, and (**c**) D3 mRNAs was analyzed by real-time RT-PCR in healthy ($n=6$), psoriatic uninvolved ($n=6$), and involved ($n=6$) epidermal samples. Values are shown as relative expression compared to healthy epidermis (mean$\pm$SD, *$P<0.05$, Student's *t*-test).

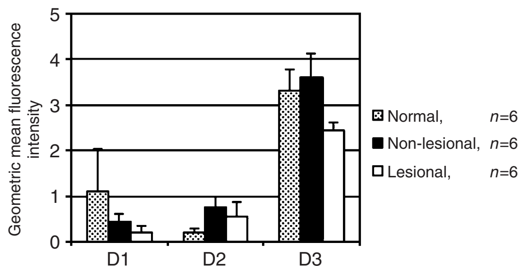


**Figure 6. Cyclin D1, D2, and D3 protein expressions in psoriatic lesional and normal epidermis.** The expression of cyclin D1, D2, and D3 proteins was analyzed by flow cytometry in healthy ($n=6$), psoriatic uninvolved ($n=6$), and involved ($n=6$) epidermal samples. The geometric mean fluorescence 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.

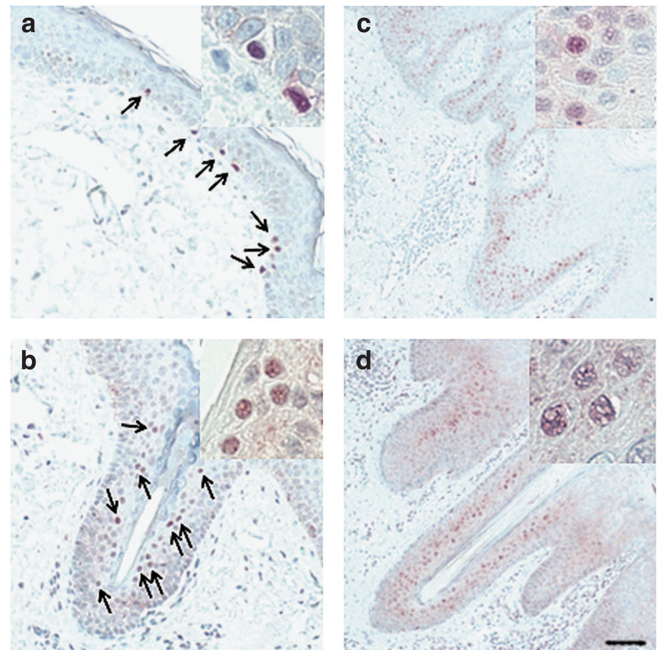


**Figure 7. Immunohistochemical detection of cyclin D1 and D3 proteins in healthy and psoriatic lesional epidermis.** (**a**) Cyclin D1-positive cells are rare, and show distinct nuclear staining in the basal layer of normal epidermis. (**c**) Intense cyclin D1 nuclear and a slight cytoplasmic staining can be detected in many more basal as well as suprabasal cells of the lesional psoriatic epidermis. Cyclin D3 staining is localized to the cytoplasm and nucleus, and detected only in the suprabasal cells of the hair follicles, both in (**b**) normal and in (**d**) psoriatic lesional skin. Bar$=50\,\mu$m.

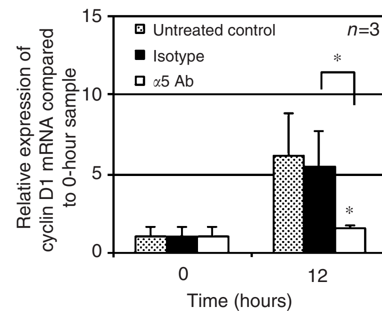


**Figure 8. Blocking the function of α5-integrin immediately after surface attachment in synchronized HaCaT keratinocytes leads to the downregulation of cyclin D1 mRNA expression.** A neutralizing monoclonal antibody to human α5-integrin was added to already attached, synchronized HaCaT keratinocytes 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 0 hour untreated sample ($n=3$) (mean$\pm$SD, *$P<0.05$, Mann–Whitney *U*-test).

## DISCUSSION

D-type cyclins are rate-limiting factors of the $G_1$ 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 (Xu *et al.*, 2003) 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 (Bartkova *et al.*, 1998). In normal human keratinocyte cultures, when quiescent ($G_0$) 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 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 large 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 $G_0$ quiescent phase and cyclin D2 and D3 expressions 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 (Robles *et al.*, 1998). 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 (Carthon *et al.*, 2005). Our results also indicate that cyclins D2 and D3 could substitute for D1 in driving keratinocyte cell cycle.

Increasing data suggest that D-type cyclins have multiple functions besides cell-cycle regulation. Cyclin D3 was ubiquitously expressed in 70 different human cultured cell types, whereas D1 and D2 were not. Complementary analyses of human and mouse tissues at different stages of fetal 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 (Bartkova *et al.*, 1998). 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 (Pivarcsi *et al.*, 2001). 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* (Burnworth *et al.*, 2006).

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 (Bata-Csorgo *et al.*, 1993), 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 expressions 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 cyclins D1, D2, and D3 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$1 integrin$^+$ K1/K10$^-$ basal compartment did not result in an overall increase in the size of this cell compartment, indicating that the proportion of $\beta$1 integrin$^+$ K1/K10$^-$ cells that self-renew (to $\beta$1-integrin K1/K10$^-$ cells) is the same in psoriatic epidermis as it is in normal epidermis, and that excess cells are committed toward differentiation (Bata-Csorgo *et al.*, 1993).

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.*, 2003) found that cyclins 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 (data not shown).

Besides transcriptional and post-transcriptional regulation, the expressions of D-type cyclins are regulated by targeted degradation via the ubiquitin pathway (Diehl *et al.*, 1998; Chung *et al.*, 2006; Okabe *et al.*, 2006; Yang *et al.*, 2007). The fact that there are more cyclin D1-positive cells in the stable psoriatic lesion localized both in the basal and 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, which 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$1-integrin$^{+}$ 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* (Yamamoto *et al.*, 2002). 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 post-transcriptional 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$5-integrin, is abnormally overexpressed in uninvolved keratinocytes of the *in vivo* psoriatic epidermis (Bata-Csorgo *et al.*, 1998) and the A domain of fibronectin (EDA$^{+}$) is present at the basement membrane zone of uninvolved psoriatic skin while absent in normal control skin (Ting *et al.*, 2000). Upon *in vitro* activation there is enhanced EDA$^{+}$ fibronectin expression in psoriatic uninvolved keratinocytes compared to normal cells (Szell *et al.*, 2004). 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 (Wang *et al.*, 2001).

We conclude that the regulatory functions of D-type cyclins in keratinocytes are not completely redundant. Although knocking down cyclin D1 in quiescent keratinocytes does not result in immediate changes in cell morphology and proliferation, both the differential expression of these cyclins in cultured keratinocytes and their abnormal expression in stable plaques of psoriasis suggest that they may have other specific functions besides driving the cell cycle in the cells. Our data also suggest a possible role for D-type cyclins in the excessive basal-cell proliferation and perturbed keratinocyte differentiation that occurs in the psoriatic epidermis. In preliminary experiments, we have 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 therapies in psoriasis.

## MATERIALS AND METHODS

### Cell culture

The spontaneously immortalized human keratinocyte cell line, HaCaT, kindly provided by Dr. N.E. Fusening (Heidelberg, Germany), was grown in 75 $cm^2$ cell culture flasks (Corning Incorporated, Corning, NY) and maintained in high-glucose DMEM (Gibco, Eggstein, Germany), supplemented with 10% fetal bovine serum (HyClone, Perbio, Budapest, Hungary), L-glutamine, and antibiotic/antimycotic solution containing 100 U/ml penicillin, 100 $\mu$l/ml streptomycin, and amphotericin B (Sigma, Steinheim, Germany) at 37°C in a humified atmosphere containing 5% $CO_2$. The medium was changed every second day.

### 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.

### 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 168 hours after the end of the synchronization process.

### Real-time RT-PCR

Total RNA was isolated from epidermis obtained from shave biopsies after Dispase digestion and from HaCaT keratinocytes using TRIzol™ reagent (Gibco, Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA concentration was determined by measuring the $A_{260}$ values. First-strand cDNA was synthetized from 1 $\mu$g 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 Bio-Rad. Primers specific cyclin D1 (cat. no. Hs 00277039), D2 (cat. no. Hs 00153380), and D3 (cat. no. 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: CGGCTACCACATCCAAG GAA, 18S RNA reverse: GCTGGAATTACCGCGGCT, 18S RNA TaqMan probe: TexRed-TGCTGGCACCAGACTTGCCCTC-BHQ-1 (Integrated DNA Technologies, Coralville, IA).

### Flow cytometric analysis of freshly separated epidermal cells and HaCaT keratinocytes

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 monoclonal antibodies used were as follows: 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 $IgG_{2a}$, and mouse $IgG_{2b}$ at the same concentrations served as isotype controls (all from Sigma). Secondary antibodies were anti-mouse IgG1, $IgG_{2a}$, and $IgG_{2b}$ coupled with fluorescein isothiocyanate, used at 1:50 and mouse $IgG_{2b}$ at 1:500 dilutions all were from BD Pharmingen). Cells (500,000) were washed in PBS containing 0.5% BSA (Sigma) and 0.1% Triton-X (Reanal), and then incubated with the primary antibody at 4°C overnight. After being washed in PBS containing 0.1% Triton-X, cells were incubated for 30 minutes at room temperature with the fluorescein isothiocyanate-conjugated secondary antibodies. 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 substraction (GeoMean of the antigen-specific antibodies stained cells – GeoMean of isotype-matched control antibodies stained cells), or as percentage of positive cells.

### Gene silencing by siRNA

HaCaT keratinocytes were contact-inhibited for 1 day and serum-starved for 5 days. Cells were then harvested and nucleofected with cyclin D1 siRNA by Nucleofector™ II (Amaxa Biosystems, Koeln, Germany) using V-Kit optimized for HaCaT keratinocytes (VCA-1003; Amaxa Biosystems) in suspensions and then plated to 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) and control siRNA (sc-36869) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and were used following the manufacturer's instructions. Samples were taken at 24, 48, and 72 hours after 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). Real-time RT-PCR was carried out as described above to quantify the mRNA expression of cyclins D1, D2 and D3. The transfection efficacy was detected using a plasmid encoding the enhanced green fluorescent protein (Amaxa Biosystems) 48 hours post-transfection by flow cytometry.

### 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 $\mu$l complete high-glucose DMEM medium for 24, 48, and 72 hours. Thiazolyl blue tetrazolium bromide assays were carried out as described previously (Sonkoly *et al.*, 2005).

### 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 minutes at 700 W, during which the incubation solution boiled for about 25 minutes, slides were rinsed with Tris-buffered saline (Sigma), containing 0.1% Triton-X (Reanal) for 15 minutes. Nonspecific staining was prevented by preincubation with 0.5% bovine serum albumin diluted in Tris-buffered saline (Sigma), containing 0.1% Triton-X (Reanal) for 30 minutes at room temperature in a humid chamber. Cells were then incubated overnight at 4°C in a humid chamber with the primary antibodies. Purified monoclonal antibodies to cyclins D1, D3 (NeoMarkers, Fremont, CA), and D2 (Fitzgerald Industries Int. Inc., Concord, MA, USA) were applied at 1:200, 1:25, and 1:2 dilutions, respectively, rabbit IgG (NeoMarkers), mouse IgG1, and mouse $IgG_{2a}$ (Sigma) 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 cyclins D2 and D3 all at 3.3 $\mu$g/ml concentration) for 1 hour at room temperature, followed by incubation with horse radish peroxidase-conjugated streptavidin for 1 hour at room temperature (Vectastain ABC Kit; Vector, Burlingame, CA), and finally peroxidase activity was detected using 3,3 amino-9 ethylcarbazole (Sigma) as a substrate. Slides were counterstained with hematoxylin (Sigma). Tissue staining was visualized using a Zeiss Axio Imager microscope and photographed using a PixeLINK digital camera.

### α5-integrin blocking assay

A total of $1 \times 10^6$ synchronized HaCaT keratinocytes were seeded into 25 $cm^2$ cell culture flasks (Sarstedt, Germany). Five hours after seeding, when the cells were 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) was added to the cells at a final concentration of 20 $\mu$g/ml. Mouse IgG1 (BD Pharmingen) at identical concentration was used for isotype control. HaCaT keratinocytes were incubated with the antibody for 7 hours. Samples were collected before (0 hour) and 12 hours after the seeding of the cells. Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Samples were analyzed by real-time RT-PCR to quantify the mRNA expression of cyclins D1, D2, and D3.

### Data presentation

Gene expression results were expressed as fold increases over control values. Data were presented as mean ± SD for *n* experiments. Flow cytometry data were expressed either as percentage 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 followed by Dunnett's *post hoc* test to determine statistical differences after multiple comparisons (SPSS, SPSS Inc., Chicago, IL). Data from the α5-integrin blocking assay were compared using the Mann–Whitney *U*-test. *P*- values of less than 0.05 for both parametric and non-parametric tests were considered significant.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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