Expression of 25-Hydroxyvitamin D-1α-Hydroxylase (1αOHase, *CYP27B1*) Splice Variants in HaCaT Keratinocytes and Other Skin Cells: Modulation by Culture Conditions and UV-B Treatment *In Vitro*

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Abstract. *In vitro and in vivo studies have demonstrated that the biologically active vitamin D metabolite 1,25-dihydroxyvitamin D (1,25(OH)$_2$D$_3$, calcitriol) suppresses proliferation and induces differentiation in various cell types, including human keratinocytes and melanocytes. Vitamin D is synthesized in the skin from 7-dehydrocholesterol (7-DHC) by the action of UV-B. There are two principal enzymes involved in the formation of circulating 1,25(OH)$_2$D$_3$ from vitamin D, the hepatic microsomal or mitochondrial vitamin D-25-hydroxylase (CYP27A1) and the renal mitochondrial 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) for vitamin D and 25(OH)D$_3$ (calcidiol), respectively. Extrarenal activity of CYP27B1 has been reported in various cell types including macrophages, keratinocytes, prostate and colon cancer cells. It has been speculated that the extrarenal CYP27B1-mediated local synthesis of 1,25(OH)$_2$D$_3$ represents a key regulator of important cellular functions including growth and differentiation in various cell types and tissues by autocrine or paracrine signalling pathways. The keratinocyte represents the only cell type where the complete enzymatic machinery for the synthesis of 1,25(OH)$_2$D from 7-DHC is present and where the synthesis of 1,25(OH)$_2$D$_3$ from 7-DHC has been shown. In this study, we characterized the pattern of CYP27B1 splice variants in HaCaT keratinocytes in vitro. Applying nested touch-down PCR, the full length CYP27B1 gene product and several additional CYP27B1 splice variants were detected. The pattern of CYP27B1 splice variants varied depending on the cell density, the calcium concentration of the medium ([Ca^{2+}]$_o$), and UV-B treatment. It can be speculated whether increased expression of CYP27B1 splice variants that lack enzymatic activity (Hyd-V3/V5) may result in a reduction of enzymatic activity and in reduced synthesis of 1,25(OH)$_2$D$_3$. Further study of the impact of CYP27B1 splice variants on the vitamin D pathway in keratinocytes and other cell types is warranted.*

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Vitamin D is synthesized in the skin from 7-dehydrocholesterol (7-DHC) by the action of UV-B. It is now well known that 1,25-dihydroxyvitamin D (1,25(OH)$_2$D$_3$, calcitriol) is the biologically active vitamin D metabolite. There are two principal enzymes involved in the formation of circulating 1,25(OH)$_2$D$_3$ from vitamin D, the hepatic microsomal or mitochondrial vitamin D-25-hydroxylase (CYP27A1) and the renal mitochondrial 25-hydroxyvitamin D-1α-hydroxylase (1αOHase, CYP27B1) for vitamin D and 25(OH)D$_3$ (calcidiol), respectively (1). These hydroxylases belong to a class of proteins known as cytochrome P450 mixed function monooxidases. Interestingly, extrarenal activity of CYP27B1 has been reported in various cell types including macrophages, keratinocytes, prostate and colon cancer cells (2). In this context, the keratinocytes are the only cell type where the complete enzymatic machinery for the synthesis 1,25(OH)$_2$D from 7-DHC is present and where the synthesis of 1,25(OH)$_2$D$_3$ from 7-DHC has been shown (3). It has been speculated that the extrarenal CYP27B1-mediated local synthesis of 1,25(OH)$_2$D$_3$ represents a key regulator of important cellular functions including growth and differentiation in various cell types and tissues by autocrine or paracrine signalling pathways (4).

In vitro and *in vivo* studies have demonstrated that 1,25(OH)$_2$D$_3$ suppresses proliferation and induces differentiation in various cell types, including human keratinocytes and melanocytes (5-9). It is generally accepted that the antiproliferative effect is the result of a vitamin D receptor (VDR)-mediated action on the genome (10, 11). Consequently, it has been speculated that vitamin D analogs

may represent promising new agents for the treatment of malignancies, including malignant melanoma and other types of skin cancer (12). Keratinocytes express VDR and represent a key target for physiological or pharmacological effects of $1,25(OH)_2D_3$ (13). However, the therapeutical use of systemically applied vitamin D analogs for treating cancer has not yet fulfilled its promise (14). A main reason for these disappointing results is that their use is limited by severe side-effects, mostly hypercalcemia, at the supraphysiological doses needed to reach clinical improvement (14). Thus, new strategies that may enable the use of vitamin D analogs that exert selective (growth-inhibitory activity combined with a reduced effect on calcium and bone metabolism) or tissue-specific activity are employed at the moment. Several of these new concepts are based on recent laboratory results demonstrating that various cell types have the capacity for tissue-specific local production of calcitriol (12).

It has previously been described for other cytochrome P450 genes (15) that alternative splicing can play a role in regulating the enzyme level and may cause tissue-specific variations in healthy cells. Moreover, numerous studies have shown that alternative splicing occurs frequently in human cancer cells (*e.g.* breast and ovarian cancer and glioblastoma multiforme; 16-18). We have previously reported the expression of *CYP27B1* splice variants in various cell types, including skin cells (19). Furthermore, using Western analysis and polyclonal antibodies, seven different proteins that, due to their molecular weight, most likely represent CYP27B1 variants Hyd-V1–Hyd-V5, Hyd-V9 and Hyd-V16 first isolated and sequenced in glioblastoma cells were detected (19, 20). The aim of this study was to further investigate the role of *CYP27B1* splice variants in skin cells. The following questions were addressed: Does UV-B-treatment modulate the *CYP27B1* splicing variant pattern in human keratinocytes *in vitro*? Do variations in cell culture conditions influence the *CYP27B1* splicing variant pattern in human keratinocytes *in vitro*? Do the *CYP27B1* splicing variant pattern occur in malignant compared with normal skin cells?

Materials and Methods

Cell culture. The spontaneously immortalized human keratinocyte cell line HaCaT (21) was maintained in DMEM medium (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 25 mM HEPES, 1% L-glutamine and 10% fetal bovine serum (FBS, PAA Laboratories GmbH). For the cultivation of the cells under low calcium concentration (0.15 mM) conditions, the medium was changed to DMEM low calcium (PAA Laboratories GmbH). The human melanoma cell lines MeWo (22) and SkMel5 (22), as well as the human cutaneous squamous cell carcinoma cell line SCL-1 (23) were cultivated in RPMI-1640 (PAA Laboratories GmbH) with 10% FBS. SCL-1 cells were obtained from the tumor and cell line bank of the Dermatology Clinic of the Saarland University Hospital. Melanocytes were isolated from foreskins (kindly provided by the Clinic of Urology and Pediatric Urology, University of the Saarland, Homburg/Saar, Germany) as published previously (24) and were cultured *in vitro* for several weeks. After enzymatic digestion, an epidermis cell suspension was cultured in melanocyte growth media (basal medium plus growth factors; PromoCell GmbH, Heidelberg, Germany). The growing melanocytes were routinely cultivated as monolayers at 37˚C and in a 5% CO_2 atmosphere. The immortalized human sebaceous gland cell (sebocyte) line SZ95 (25) was a gift from C.C. Zouboulis (Dessau, Germany). The SZ95 cells were grown in Sebomed basal medium (Biochrom, Berlin, Germany) containing 10% heat inactivated FBS and 5 ng/ml human recombinant epidermal growth factor. All the cells were cultivated at 37˚C in 5% CO_2 using 75 cm^2 cell culture flasks or 96-well plates (Nunc, Naperville, IL, USA).

UVB irradiation of cultured cells in vitro. To analyze the UV-B-mediated effects on the expression of *CYP27B1* splice variants, semi-confluent and confluent HaCaT cells were treated with UV-B at various doses (7.5 mJ/cm^2, 10 mJ/cm^2, 20 mJ/cm^2 or 50 mJ/cm^2). The medium was removed from 70-80% confluent cell cultures, the cells were rinsed with PBS and exposed to UVB using a Waldmann W lamp (UV 409T, Waldmann Lichttechnik, Villingen-Schwenningen, Germany), then the culture medium was replaced. The UVB dose was determined using a Waldmann UV-meter (type 585100 Waldmann Lichttechnik).

Pre-incubation with 7-dehydrocholesterol (7-DHC) before UVB exposure. 7-DHC (25 μM; Sigma-Aldrich Chemie, Taufkirchen, Germany) was dissolved in ethanol (0.5 vol %) before being added to the growth medium that had previously been supplemented with BSA (1 vol. % Bovine albumin Fraction V Solution 7.5%, Sigma-Aldrich Chemie). After a pre-incubation period of 1 h, the keratinocytes were exposed to UVB and incubated as described above. HaCaT keratinocytes were treated with 7-DHC (25 μM) 24 h prior to UV-B treatment; cells were then treated with 20 J/m^2 UV-B and harvested after 12 h.

Analysis of cell proliferation. Cell proliferation was quantified by crystal violet (CV) dye staining as previously described (26). Briefly, the cells were washed once with PBS and fixed with ethanol (70%) for 30 min at room temperature. The cells were then incubated with a CV solution (1% w/v in 20% ethanol) for 30 min at room temperature and rinsed with water thoroughly. After drying, the dye was extracted with 70% ethanol and its absorbance determined at 550 nm using a microplate reader. The antiproliferative effect of the vitamin D analogs was calculated as follows: inhibition of proliferation (%) = (1 – [CV staining in treated wells/CV staining in control wells]) ×100. The control wells were treated identically to the wells containing vitamin D analogs (treated wells) except for the addition of the vitamin D analogs.

RNA and poly (A)-RNA isolation. The total RNA from the HaCaT cells was extracted with RNeasy (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The total RNA amount was quantified spectrophotometrically and its integrity was verified by 1% agarose gel electrophoresis in 1x MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 6.3). Subsequently, 150 μg of the total RNA were used to isolate poly (A)-RNA according to the manufacturer's instructions (Oligotex mRNA, Qiagen). We previously developed a highly-sensitive nested touch-down PCR technique for the analysis of CYP27B1 splice variants

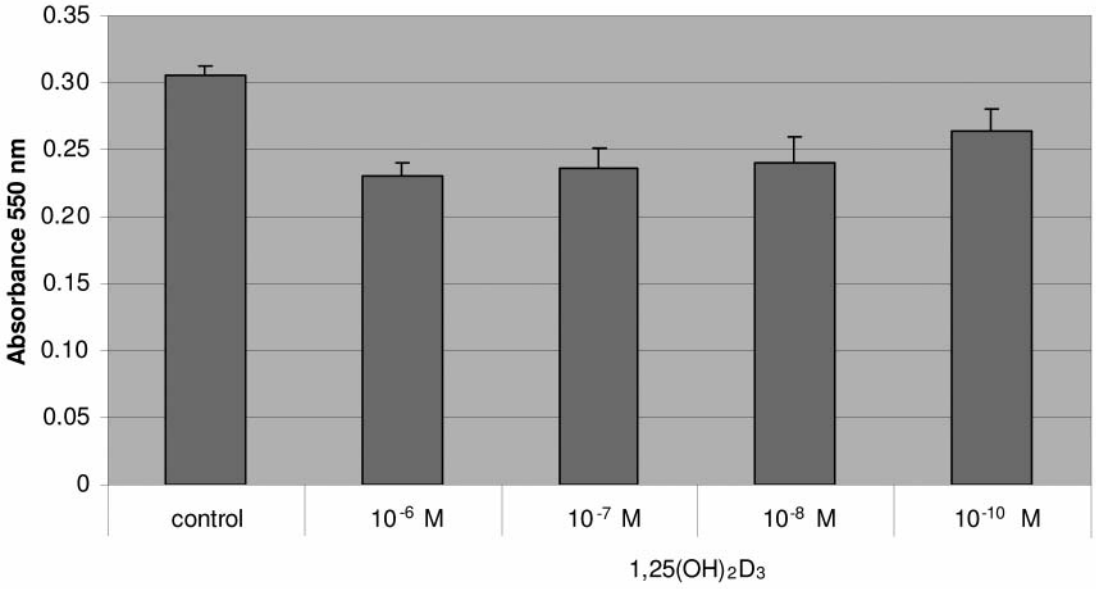

Figure 1. *Effect of 1,25(OH)$_2$D$_3$ on proliferation of HaCaT cells. Proliferation assay was performed using crystal violet dye.*

(19). Prior to its utilization, the RNA was DNase I (Invitrogen, Karlsruhe, Germany)-treated. First-strand cDNA was synthesized with Onmiscript reverse transcriptase (Qiagen) and oligo-d(T)15 primer (Invitrogen). The first PCR was performed using primers *Sp1a*For1 (5'-GGAGAAGCGCTTTCTTTCG-3') and *Sp1a*Rev3 (5'-TGGGGCAAACCCACTTAATA-3') with 10 cycles (2 min at 98˚C, 15 s at 94˚C, 20 s at 68˚C, 4 min at 68˚C, 15 min at 68˚C). The PCR product was purified (Nucleo Spin Extract II, Machery-Nagel, Düren, Germany) and 5 µl was used as the template for the second PCR using primers HE1 (5'-CAGACCCTCAAGTACGCC-3') and *Sp1a*Rev2 (5'-AAACCAGGC TAGGGCAGATT-3'). This PCR consisted of 12 cycles (30 s at 96˚C, 10 s at 94˚C, 20 s with a touchdown from 68˚C to 62˚C in 0.5˚C intervals, 4 min at 68˚C) followed by 18 cycles (10 s at 64˚C, 20 s at 62˚C, 4 min at 68˚C). The PCR reactions were performed using 2.5 Units RedACCu Taq™ LA DNA polymerase (Sigma Aldrich, Munich, Germany). The obtained PCR products were separated on a 1% agarose gel.

Effect of cell density and calcium concentration on expression of CYP27B1 splice variants. HaCaT cells were seeded at 40% confluency and cultured for 6 days. On each day, a fraction of cells was harvested, proteins were isolated and *CYP27B1* splice variants were investigated using Western blot analysis. HaCaT keratinocytes where also cultured for one day in DMEM containing high calcium (1.5 mM). On the next day, the medium was changed and the HaCaT cells were cultivated for 48 h with medium containing low calcium (0.15 mM). The cells were harvested at various time points and mRNA and protein expression were analyzed.

Real-time PCR for expression analysis of VDR, CYP27A1, CYP27B1 and CYP24A1 in HaCaT keratinocytes. The expressions of *VDR, CYP27A1, CYP27B1,* and *CYP24A1* (24-hydroxylase, 24-OHase) were analyzed in HaCaT cells using real-time PCR (LightCycler, Roche, Mannheim, Germany, 50 cycles) and gene-specific primers as published previously (27).

The relative amounts of the target genes and a reference gene (β2-microglobulin) were calculated for each sample and one calibrator, integrated in each Lightcycler run. The relative ratio of the target to reference for each sample and for the calibrator was calculated first. This corrected for sample-to-sample variation caused by differences in the initial quality and quantity of the nucleic acid (Roche, technical note LC 13/2001) The target/reference ratio of each sample was then divided by the target/reference ratio of the calibrator using relative quantification software (Relquant; Roche Molecular Biochemicals, Mannheim, Germany). This second step normalized for different detection sensitivities of the target and reference amplicons. Thus, the normalization to a calibrator provided a constant calibrator point between the PCR runs. The experiments were conducted in duplicate and the final results were expressed as the median of the N-fold differences in target gene expression in treated cells relative to the normal cells.

Western blot analysis. The cells were harvested, washed twice with PBS and lysed in sample buffer (125 mM Tris, 30% glycerine, 8% SDS, pH 6.8) and 20 µg of proteins were subjected to 12.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and after separation transferred to a nitrocellulose membrane (Optitran BA-S 85; Schleicher Schuell, Dassel, Germany). The membranes were blocked with TBST (25 mM Tris-Cl, pH 8; 125 mM NaCl, 0.1% Tween) containing 5% non fat powdered milk and 0.1% Tween at room temperature and were incubated h with primary antibodies against human 1αOHase at a dilution of 1:2,000 overnight at 4˚C. The primary antibody was generated and purified as described previously (15, 16) The secondary antibodies conjugated to horseradish peroxidase (anti-mouse IgG, Amersham Biosciences, Freiburg, Germany) were added in a dilution of 1:6,000. After several washing steps, the bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). The obtained signals were compared to β-actin as the internal standard.

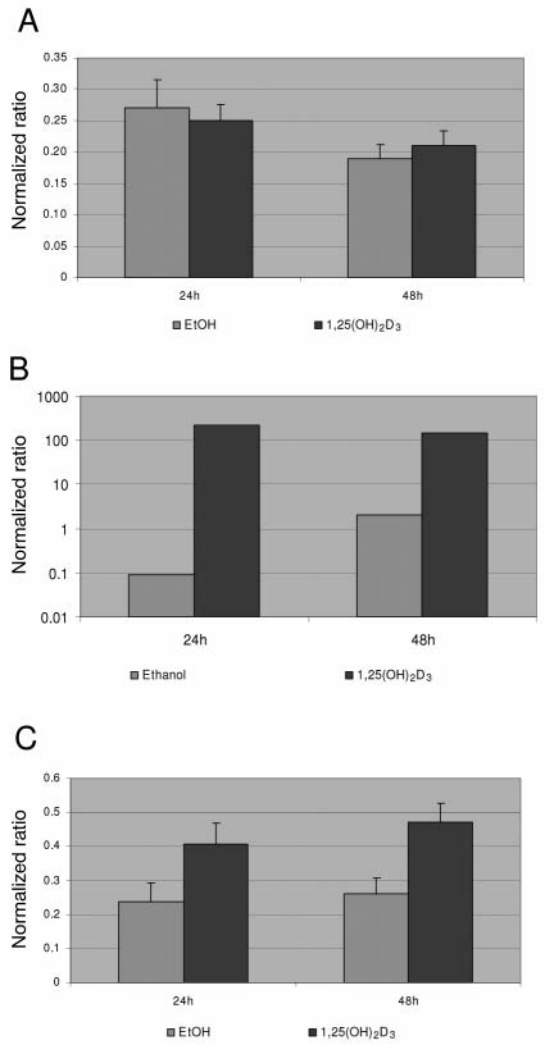

Figure 2. *Expression of CYP27B1 (A), CYP24A1 (B), and VDR (C) mRNA in 1,25(OH)$_2$D$_3$-treated HaCaT cells.*

Statistical analysis. The proliferation data are expressed as mean ± S.E. Each data point represents the mean of three separate experiments. One-way analysis of variance (ANOVA) was used to assess the statistical significance between individual means in the treated HaCaT cells as compared to controls. The cut-off point for significance was defined as p=0.05.

Results

Proliferation of HaCaT keratinocytes. Treatment of the HaCaT keratinocytes with 1,25(OH)$_2$D$_3$ resulted in a dose-dependent inhibition of cell proliferation *in vitro*, as measured by the crystal violet dye assay (Figure 1). After 72-h treatment with 1,25(OH)$_2$D$_3$ at 10^{-6} M and 10^{-10} M, proliferation was inhibited by 26% and 15% respectively, as compared to the control.

Expression of CYP27A1, CYP27B1 and CYP24A1 in HaCaT keratinocytes. The key enzymes involved in the synthesis (CYP27A1, CYP27B1) and metabolism (CYP24A1) of 1,25(OH)$_2$D$_3$ were completely expressed in the HaCaT keratinocytes. Treatment with 1,25(OH)$_2$D$_3$ (10^{-6} M) resulted in a 1,000-fold (0.1 to 100 of normalized ratio) increase of *CYP24A1*, the classical vitamin D-responsive target gene, thereby indicating the functional integrity of VDR-mediated gene transcription in the HaCaT keratinocytes. While treatment with 1,25(OH)$_2$D$_3$ resulted in a 2-fold increase in *VDR* expression, no modulation of *CYP27B1* expression was found (Figure 2).

Analysis of CYP27B1 splice variants by nested touch-down PCR. The full length *CYP27B1* gene product and several additional *CYP27B1* splice variants were detected (Figure 3). Comparing the *CYP27B1* splicing variant pattern in the HaCaT keratinocytes with that in other tissues and cell lines, the pattern in the HaCaT cells was comparable to the pattern characteristic of the kidney but was remarkably different as compared to the normal melanocytes and the melanoma cells (Figure 3). In SZ95 sebocytes, the predominant transcript is the full length *CYP27B1* gene product. The bands identified in the agarose gel showed several fragments which were bigger than the fragment of the full length product. The size of the amplified fragments suggests that several variants were expressed which contained the intron 1 and represented the *CYP27B1* splice variants HydV.6 to HydV15 that we have characterized previously (20).

Western blot analysis of CYP27B1 splice variants. Three strong bands were detected in the HaCaT keratinocytes and all the other cell lines analyzed (SZ95, SCL-1, normal melanocytes, MeWo, SK-Mel-5). These bands corresponded to proteins with a molecular weight between 45 and 60 kDa. The individual cell lines differed in the intensity of the smaller variants. Interestingly, a marked difference could be seen when the normal melanocytes were compared with the melanoma cell lines. While the individual bands showed a relatively constant intensity in the melanoma cell lines (*e.g.* Sk-Mel-5), the full length product representing the active form of *CYP27B1*, exerted a markedly pronounced intensity as compared with the two smaller and inactive variants Hyd-V4 and Hyd-V3/V5 in the normal melanocytes (Figure 4).

Expression of CYP27B1 splice variants in semi-confluent vs. confluent HaCaT keratinocytes. After four days, the cells reached confluency and started floating off. As shown in Figure 5, there were only marginal differences in the *CYP27B1* splicing variant pattern at the different time points. The semi-confluent, proliferating HaCaT cells (day 3) strongly expressed three bands that most likely corresponded to the full length

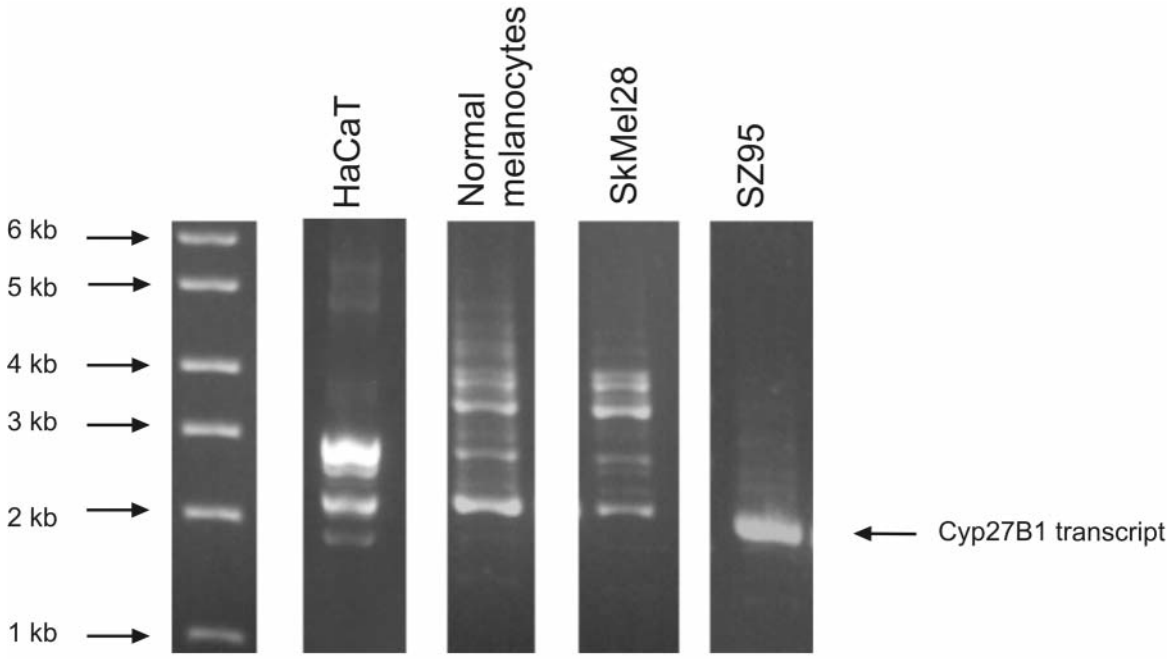

Figure 3. *Analysis of CYP27B1 splice variants in skin cells using nested touch-down PCR. With the exception of Hyd-V4, all CYP27B1 splice variants that represent longer transcripts as compared to the full length CYP27B1 transcript contained intron 1 and did not generate a continuous reading frame.*

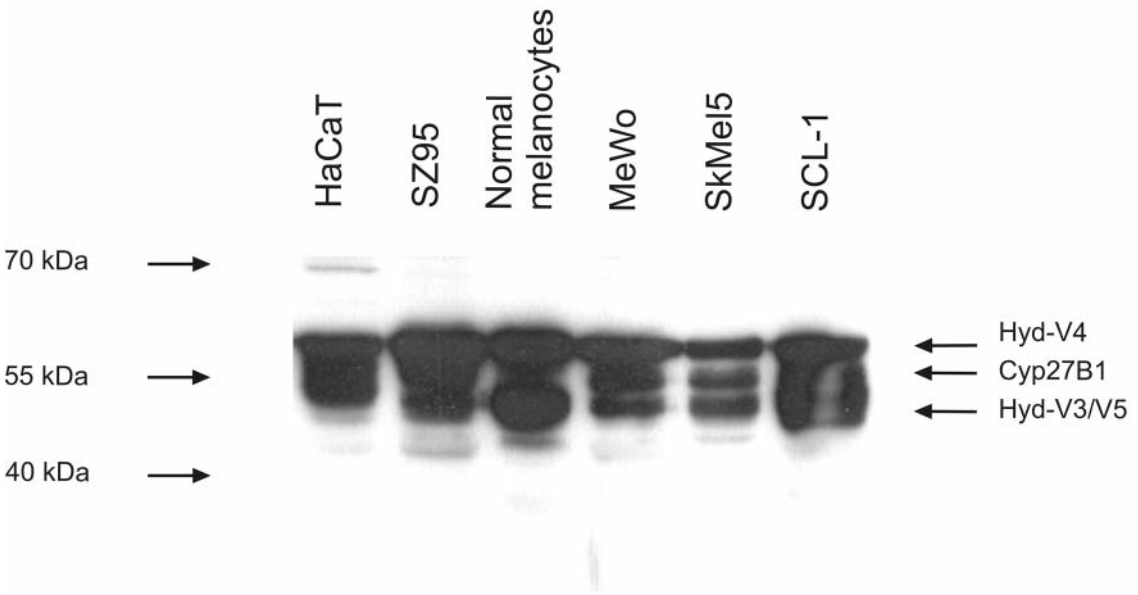

Figure 4. *Western blot analysis of CYP27B1 splice variants in different skin cell types (HaCaT keratinocytes, SZ95 sebocytes, normal melanocytes, MeWo and SkMel5 melanoma cells, SCL-1 cutaneous squamous carcinoma cells).*

CYP27B1 gene transcript and variants Hyd-V4 and Hyd-V3/V5. However, the confluent HaCaT cells (day 6) showed only a weak and almost undetectable band corresponding to Hyd-V3/V5. No differences were found when the two other bands were compared at individual time points.

Expression of CYP27B1 splice variants in HaCaT keratinocytes cultivated in high vs. low calcium conditions. Interestingly, the HaCaT cells grown for 12 h in medium containing a low calcium concentration (0.15 mM) show markedly reduced bands corresponding to the full length

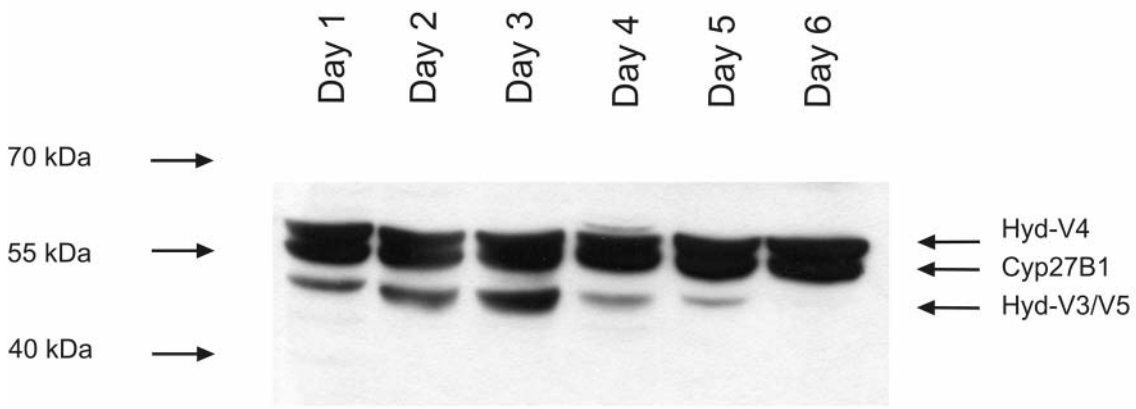

Figure 5. *Cell density-dependent variations of the CYP27B1 splicing variant pattern in HaCaT keratinocytes analyzed by Western blot analysis.*

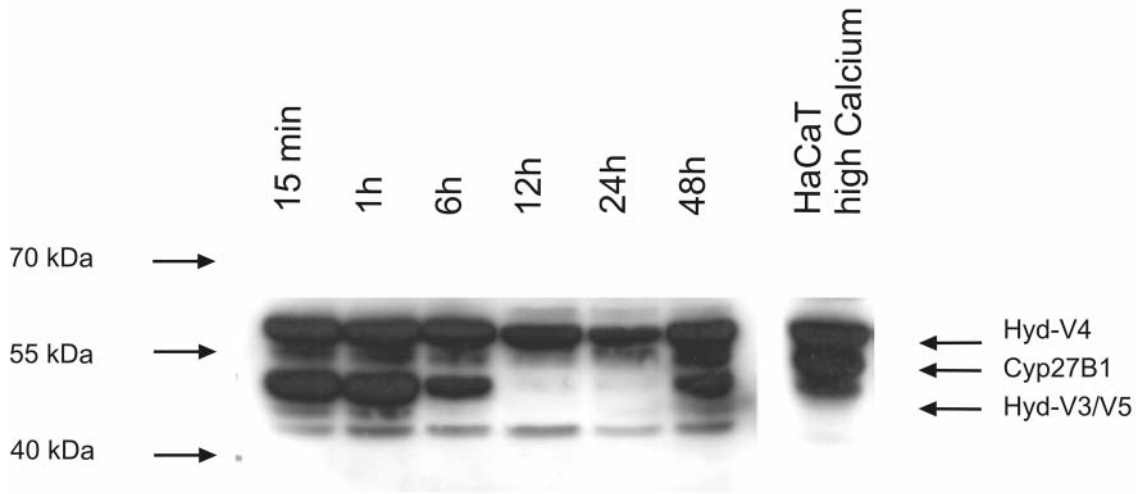

Figure 6. *Cell culture-dependent variations of the CYP27B1 splicing variant pattern in HaCaT keratinocytes.*

CYP27B1 gene transcript and variants Hyd-V3/V5 as compared to the HaCaT cells grown in medium containing 1.5 mM calcium (Figure 6). After 48 h of culture, the *CYP27B1* splicing variant pattern in the cells cultured in medium containing low calcium (0.15 mM) was comparable with that from the cells cultured in medium containing high (1.5 mM) calcium. The *CYP27B1* splicing variant pattern of the semi-confluent HaCaT cells grown for 12 h in medium containing 1.5 mM calcium was comparable to the pattern in the confluent HaCaT cells grown for 48 h in medium containing low calcium concentration (0.15 mM).

Western analysis of CYP27B1 splice variants in HaCaT keratinocytes following UV-B treatment. In the semi-confluent (approx. 80%) and rapidly proliferating cells, UV-B-treatment did not modulate the expression pattern at any dose or time-point analyzed using Western blot analysis (data not shown). In contrast, UV-B treatment of the confluent and more differentiating HaCaT cells resulted in appreciable time- and dose-dependent change. Additional bands were found following UV-B treatment (Figure 7), with a striking difference between the cells treated with 7.5 mJ/cm^2 or 10 mJ/cm^2 and those treated with 20 mJ/cm^2 or 50 mJ/cm^2. A new band of approx. 45 kDa was observed at a dose of 20 or 50 mJ/cm^2 after 6 h. The original three fragments were reduced in their intensity or no longer demonstrable after 12 h.

Western blot analysis of CYP27B1 splice variants in HaCaT keratinocytes following treatment with 7-DHC and UV-B. No visual modification of *CYP27B1* splicing variant pattern was found using Western blot analysis in the 7-DHC treated compared to the untreated cells after UV-B irradiation

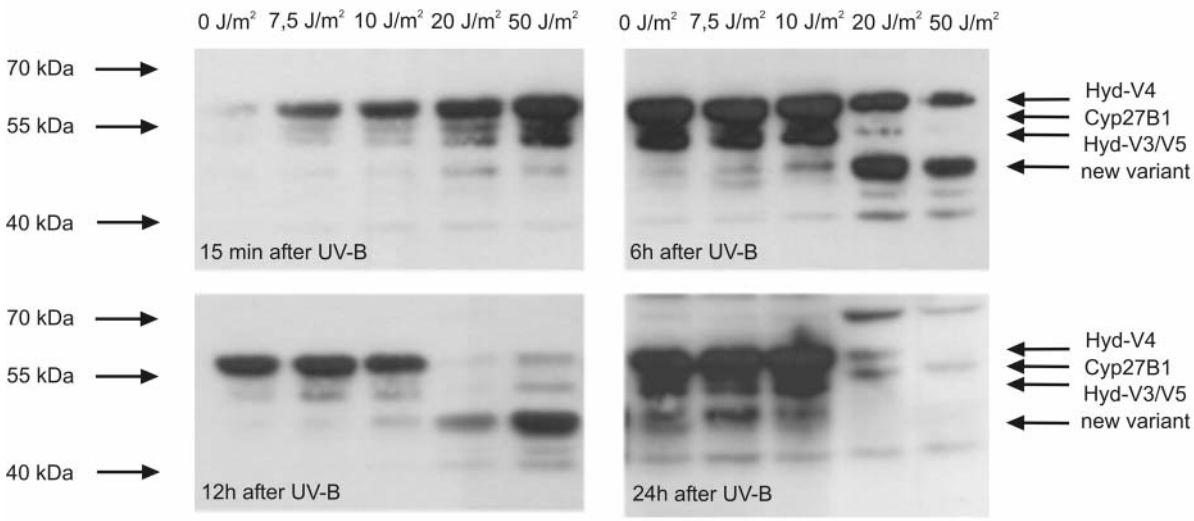

Figure 7. *Dose- and time-dependent UV-B-induced modulation of expression of CYP27B1 splice variants in HaCaT keratinocytes. Western blot analysis shows induction of a relatively small CYP27B1 variant indicated by an arrow 6 h after UV-B-treatment with 20 or 50 J/m² associated with reduced expression of the full length CYP27B1 gene transcript and some splice variants.*

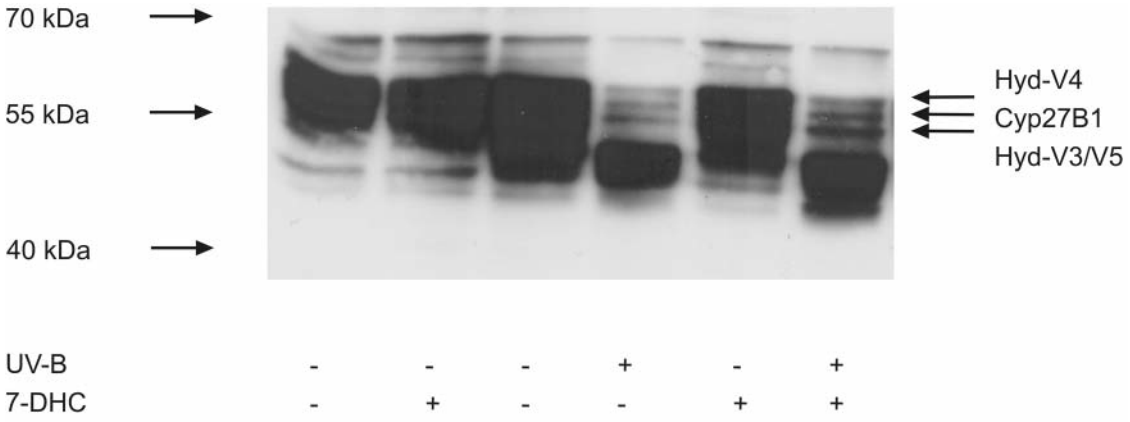

Figure 8. *Pretreatment with 7-DHC and UV-B-induced expression of CYP27B1 splice variants in HaCaT keratinocytes.*

(Figure 8). The UV-B-induced reduced expression of the full length CYP27B1 gene transcript was seen with or without pretreatment with 7-DHC.

Discussion

In this study, the previous findings that HaCaT keratinocytes possess the complete enzymatic machinery for the local synthesis of $1,25(OH)_2D_3$ (3) were confirmed the synthesis of $1,25(OH)_2D_3$ from 7-DHC was shown *in vitro*. Treatment with $1,25(OH)_2D_3$ blocked the proliferation and increased the expression of *VDR* and *CYP24A1* in the HaCaT keratinocytes. Moreover, these cells were shown to express *CYP27B1* splice variants. Three characteristic bands were detected that most likely corresponded to the full length product of *CYP27B1* (56 kDa) and splice variants Hyd-V4 (59 kDa) and Hyd-V3/V5 (46 kDa). Variant Hyd V4 is characterized by an inframe insertion of exon 2 and, with a molecular weight of 59 kDa, represents the only *CYP27B1* splicing variant reported so far that has a higher molecular weight as compared with the full length product. CYP27B1 is localized in the inner membrane of mitochondria and to fulfil its enzymatic activity, several functional domains of the CYP27B1 protein are necessary, including a ferredoxin-binding site in exon 6 and a heme-binding site in exon 8. We have shown previously that most *CYP27B1* splice variants

contain an insertion in intron 1 (19). In individual splice variants, this insertion is combined with several other exon/intron variations. The insertion in intron 1 causes a dislocation of the reading frame which results in the recognition of a premature stop codon. The translation of *CYP27B1* splice variants that do not contain intron 1 but are characterized by other exon/intron variations also results in truncated proteins that lack completely or in part the protein domains that are of importance in fulfilling its enzymatic activity. Only one of the known splice variants, Hyd-V4 has the potential to encode for a protein that may possess the full enzymatic activity. This splicing variant contains an in-frame insertion of 27 amino acids. Using transient expression in COS-1 cells, a cell line derived from kidney cells of green monkeys and thin layer chromatography, we previously analyzed the enzymatic activity of *CYP27B1* splice variants (20). Expression of the full length *CYP27B1* resulted in a marked increase of enzymatic activity. In contrast, expression of truncated variants (Hyd-V2) did not result in detectable enzymatic activity (20). Expression of the only *CYP27B1* splicing variant that contains both the ferredoxin and the heme domain (Hyd-V4) also did not result in enzymatic activity (20). However, the lack of enzymatic activity does not mean that these CYP27B1 are biologically inactive. Recently, Wu and co-workers convincingly demonstrated that a truncated non coding *CYP27B1* splicing variant containing intron 2 represented a key regulator of $1,25(OH)_2D_3$ synthesis in renal HKC-8 cells, an immortalized human renal proximal tubular cell line (28). Interestingly, the treatment with siRNA directed against intron 2 of *CYP27B1* resulted in significantly increased enzymatic activity of CYP27B1 (28).

Recently, it has been shown that knock-out mice deficient for *CYP27B1* or *VDR* are characterized by alterations in epidermal differentiation (29-31). These knock-out mice showed reduced epidermal expression of involucrin, loricrin and profilagrin and a decrease in epidermal keratohyalin granulae (31). Moreover, the skin of *CYP27B1* knock-out mice exhibited a prolonged loss of barrier-function and a defective reconstitution of the epidermal calcium gradient following the induction of skin lesions (31). In contrast, the skin of *VDR* knock-out mice was not characterized by a defective barrier function (31). Moreover *CYP27B1* knock-out mice were not characterized by defective hair cycling, in contrast to *VDR* knock-out mice. These phenotypic differences of the *CYP27B1* and *VDR* knock-out genotypes may be caused by the fact that the biological effects of CYP27B1 are not restricted to the synthesis of $1,25(OH)_2D_3$. CYP27B1 may exert additional biological functions that are still unknown.

The mechanisms by which $1,25(OH)_2D_3$ induces epidermal differentiation are not completely understood and overlap with mechanisms by which calcium regulates cellular differentiation. Whether *CYP27B1* splice variants are of high importance for this process is open to speculation.

In this study, the pattern of *CYP27B1* splice variants varied depending on the cell density, the calcium concentration of the medium ($[Ca^{2+}]_o$), and UV-B treatment. Increased expression of *CYP27B1* splice variants that lack enzymatic activity (Hyd-V3/V5) may result in a reduction of enzymatic activity and in reduced synthesis of $1,25(OH)_2D_3$. Further study of the impact of *CYP27B1* splice variants on the vitamin D pathway in keratinocytes and other cell types is warranted.

The present results were only in part comparable to the recent findings of Vantigenem *et al*. (32), who reported an induction of *CYP24A1* after the UV-B treatment of dermal fibroblasts in the presence of 7-DHC. No induction of *CYP24A1* after UV-B treatment in the HaCaT cells in the presence of 7-DHC was found in the present study. This discrepancy may be in part explained by the UV-B-induced induction of inactive *CYP27B1* splice variants in HaCaT keratinocytes in the absence of 7-DHC. The addition of 7-DHC might have been been expected to influence the expression of *CYP27B1* splice variants in the HaCaT cells. However, this was not the case. Future investigations are needed to analyze which *CYP27B1* splice variants are expressed in human keratinocytes following UV-B treatment.

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