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SHORT COMMUNICATION

The expressions of ABCC4 and ABCG2 xenobiotic transporters in human keratinocytes are proliferation-related

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Abstract Xenobiotic transporters of the ATP-binding cassette (ABC) protein superfamily play important roles in maintaining the biochemical barrier of various tissues, but their precise functions in the skin are not yet known. Screening of the expressions of the known xenobiotic transporter genes in two in vitro keratinocyte differentiation models revealed that the ABCC4 and ABCG2 transporters are highly expressed in proliferating keratinocytes, their expressions decreasing along with differentiation. Abrogation of the ABCC4 and ABCG2 protein functions by siRNA-mediated silencing and chemical inhibition did not affect the proliferation of HaCaT cells. In contrast, disruption of the ABCG2 function had no effect on normal human epidermal keratinocyte proliferation, while the inhibition of ABCC-type transporters by probenecid resulted in a striking decrease in the proliferation of the cells. These results indicate that, besides their possible therapy-modulating effects, xenobiotic transporters may contribute significantly to other keratinocyte functions, such as cell proliferation.

Keywords Xenobiotic transporters · Keratinocytes · Proliferation · Differentiation · Functional inhibition

Abbreviations

ABC	ATP-binding cassette
NHKs	Normal human epidermal keratinocytes
ITGA5	Integrin alpha 5
KRT1	Keratin 1
IVL	Involucrin

Introduction

ATP-binding cassette (ABC) transporter proteins are widely expressed in all known living organisms. Their function in ATP-driven active transport is indispensable for an appropriate cellular metabolism. ABC transporters are responsible for the transport of numerous compounds across biological membranes. The ten members of the ABC transporter superfamily are known to play a considerable part in multidrug resistance and are referred to as xenobiotic transporters [16]. Earlier research focused on the roles of these transporters in chemotherapy resistance, but interesting data have recently emerged regarding their physiological functions. In general, they are involved in normal tissue protection against environmental toxins and against endogenous compounds produced in cells during normal metabolic processes or in response to stress [4, 6, 11].

The epidermis is the largest and most important mechanical and chemical permeability barrier in the body,

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and it has been suggested that xenobiotic metabolizing enzymes such as cytochrome P450 proteins and xenobiotic transporters contribute to the biochemical barrier function of the epidermis [13]. There have been only a few studies to date concerning the roles of xenobiotic transporters in normal human epidermal keratinocytes (NHKs) or in human skin. Some of the xenobiotic transporters are expressed in NHKs; as an example membrane-associated immunostaining of ABCB1 and ABCC1 has been detected in the epidermis [3]. The complete range of known ABC transporters was arrayed during in vitro Ca^{2+}-induced NHK and HaCaT keratinocyte differentiation in a study focusing on lipid trafficking during terminal differentiation of the cells [8]. The gene expression of the ABCC1 xenobiotic transporter has been investigated in detail in healthy human skin, in psoriasis and after UV light treatment [17].

The aim of the present investigation was to acquire detailed information relating to the expressions of all known human xenobiotic transporter genes in keratinocytes: ABCB1 (Pgp/MDR1), ABCC1-6 (MRP1-6) and ABCG2 (BCRP). ABCB11 (BSEP) and ABCC11 (MRP8) were not included in our experiments, since they have been reported not to be expressed in keratinocytes [8]. Systematic studies on two in vitro keratinocyte proliferation and differentiation models demonstrated a similar regulation of the ABCC4 and ABCG2 transporters in connection with cell proliferation. The involvement of the ABCC4 and ABCG2 transporters in keratinocyte proliferation was also investigated by using gene-specific silencing and chemical inhibitors.

Materials and methods

Cell culture

Normal human epidermal keratinocytes were obtained and cultured as described previously by Kinyo et al. [9]. HaCaT keratinocytes, kindly provided by Dr. N. E. Fusenig (Heidelberg, Germany), were maintained in T-75 flasks (Corning, NY, USA) in high-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (HyClone, Logan, UT, USA), 1% L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and AB/AM solution. NHKs and HaCaT cells were grown in a chamber at 37°C in a humidified atmosphere containing 5% CO_2. The culture media were changed every second day.

Keratinocyte differentiation models

HaCaT keratinocytes were synchronized by a chemical-free procedure described by Pivarcsi et al. [14].

Normal human epidermal keratinocytes were cultured until 90–95% confluence in the third passage. Culturing conditions were altered by increasing the Ca^{2+} concentration in the culture medium to 1.7 mM (in the form of $CaCl_2$), to promote terminal differentiation. Samples were taken at 0, 1, 2, 4, 6, 8 and 10 days after switching to the high-Ca^{2+} concentration medium. Samples from controls containing 0.09 mM Ca^{2+} were collected at the same time points.

Quantitative real-time RT PCR

Total RNA was isolated from cells through the use of TRIzol™ Reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time RT-PCR experiments were carried out with the Universal Probe Library system (F. Hoffmann-La Roche AG, Basel, Switzerland); assay members are listed in Online Resource 1. PCR assays were performed with an iQ5 Real-Time PCR Detection Machine (Bio-Rad). The relative mRNA levels were calculated by the $2^{-\Delta\Delta C_t}$ method [12]. The expression of each gene was normalized to that of the 18S ribosomal RNA gene.

Western blot

Cells were trypsinized and harvested by centrifugation, and the pellet was then gently resuspended in protein lysis buffer (20 mM HEPES, 150 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, 0.5% Triton X-100, 0.1% Igepal® CA-630) containing 0.5% protease inhibitor cocktail (all components from Sigma). Protein concentrations were determined with the BCA detection kit (Thermo Scientific, Waltham, MA, USA). SDS–PAGE was carried out with 40 μg protein samples, blotted onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked in Tris-buffered saline (150 mM NaCl, 25 mM Tris, pH 7.4) containing 3% non-fat dry milk powder (Bio-Rad). Rat anti-human ABCC4 (clone M4I-10; GeneTex, Irvine, CA, USA), mouse anti-human ABCG2 (clone BXP-21; Calbiochem, Gibbstown, NJ, USA) and mouse anti-human α-actin (Sigma) were diluted at 1:150, 1:500 and 1:400, respectively, and incubated overnight at 4°C. Anti-rat and anti-mouse IgG alkaline phosphatase-conjugated secondary antibodies (Sigma) were applied and the bands were visualized with SigmaFAST BCIP/NBT (Sigma).

siRNA-mediated knockdown of transporter expression

The ABCC4- and ABCG2-specific siRNA, scrambled siRNA, transfection medium and transfection reagent were

purchased from Santa Cruz. Transfection was carried out according to the manufacturer's instructions. siRNA was used at a final concentration of 50 nM. Transfection efficiency was checked by flow cytometry, using FITC-conjugated control siRNA. HaCaT keratinocytes were transfected 1 day after seeding onto culture plates.

Flow cytometry

Cells were harvested as described above, and resuspended in PBS. ABCG2 surface staining, anti-ABCG2 (1:100, clone 5D3, R&D Systems) and mouse IgG2b isotype control (Sigma) primary antibodies were applied for 45 min on ice, and the cells were washed with PBS, and stained with anti-mouse IgG Alexa647-conjugated secondary antibody (Invitrogen). Samples were analysed on a FACSCalibur flow cytometer (Becton–Dickinson); detection was performed with excitation at 635 nm (FL-4).

MTT (thiazolyl blue tetrazolium bromide) assay

Cell viability assays were carried out as described previously by Sonkoly et al. [18].

Real-time cell proliferation analysis

5×10^3 cells were seeded onto 96-well E-plates (Roche), integrated with gold microelectrode arrays, and real-time cell analysis (RTCA) was carried out with the xCELLigence System (Roche). Application of a low-voltage alternating current signal generates an electric field between the electrodes, which is modulated by the cells covering the electrodes. Cell proliferation in the wells results in changes in the impedance readout, obtained from each well with the RTCA SP Instrument. The generated signal is displayed in arbitrary units, referred to as the cell index. After a 24-h initial incubation on the E-plates, HaCaT cells and NHKs were treated with probenecid (Sigma; 0.5 mM) and Ko-134 (Solvo Biotechnology, Budapest, Hungary; 1 μM), inhibitors of ABCC4 and ABCG2, respectively. Vehicle-treated samples were used as controls. The cell index was monitored for 72 h, with measurements every 15 min.

Results

Proliferation-related expression of ABCC4 and ABCG2 transporters in synchronized HaCaT keratinocytes and NHKs

In order to investigate the physiological significance of xenobiotic transporters in keratinocytes, we screened the transcriptional regulation of the transporters in an in vitro model of keratinocyte proliferation and differentiation. The chemical-free synchronization of HaCaT keratinocytes was described and evaluated previously [14]. At the end of a 2-week period of contact inhibition and serum starvation, the HaCaT cells became highly differentiated and proliferatively inactive. Release of the cells from this quiescent state resulted in decreases in the expression of differentiation markers, and a synchronous and rapid proliferation was initiated. The expression of the transporters in this keratinocyte model system was followed for 1 week.

Gene expression data on the xenobiotic transporters are presented in Online Resource 2. The ABCC2 mRNA level was significantly increased 12 h after the release from cell quiescence; it subsequently decreased slightly, but remained relatively high until the end of the experiment. The ABCC4 and ABCG2 transporter genes were both upregulated between 24 and 72 h after the release from cell quiescence; there was then a return to the baseline as the cells became confluent (Fig. 1a).

To study the expressions of xenobiotic transporter genes in an alternative in vitro keratinocyte model, NHKs were treated with a high level of extracellular Ca^{2+} (1.7 mM) to initiate terminal differentiation, and control cells cultured under low-Ca^{2+} conditions (0.09 mM) were also followed. The differentiation of NHKs was monitored by determining the expression of proliferation-related markers such as Ki67 and integrin alpha 5 (ITGA5), and differentiation-related markers such as keratin 1 (KRT1) and involucrin (IVL). Details of the model are presented in Online Resource 3.

The transcriptional activities of the ABCB1, ABCC1-6 and ABCG2 transporter genes were screened in the in vitro NHK model system. A very moderate increase was detected in the relative level of ABCC2 mRNA, while the relative levels of ABCC4 and ABCG2 mRNA decreased progressively to about one-fifth of the initial value in the differentiating cells by day 4, and both transporter genes remained downregulated until the end of the experiment (Fig. 2a, b and Online Resource 4).

Of the examined xenobiotic transporter genes, ABCC4 and ABCG2 exhibited a characteristic proliferation-related expression pattern in both in vitro models, in synchronized HaCaT cells and in differentiating NHKs. We therefore set out to characterize these two xenobiotic transporters in detail. We first performed western blotting and immunocytochemistry experiments to see whether they are regulated similarly at the protein level. Both transporter proteins were almost undetectable in highly differentiated HaCaT cells at 0 h. The ABCC4 protein was clearly elevated at 36 h after the release from cell quiescence and remained overexpressed up to 96 h. The ABCG2 protein level increased sharply up to around 36 h; a gradual decrease was subsequently observed (Fig. 1b). In the

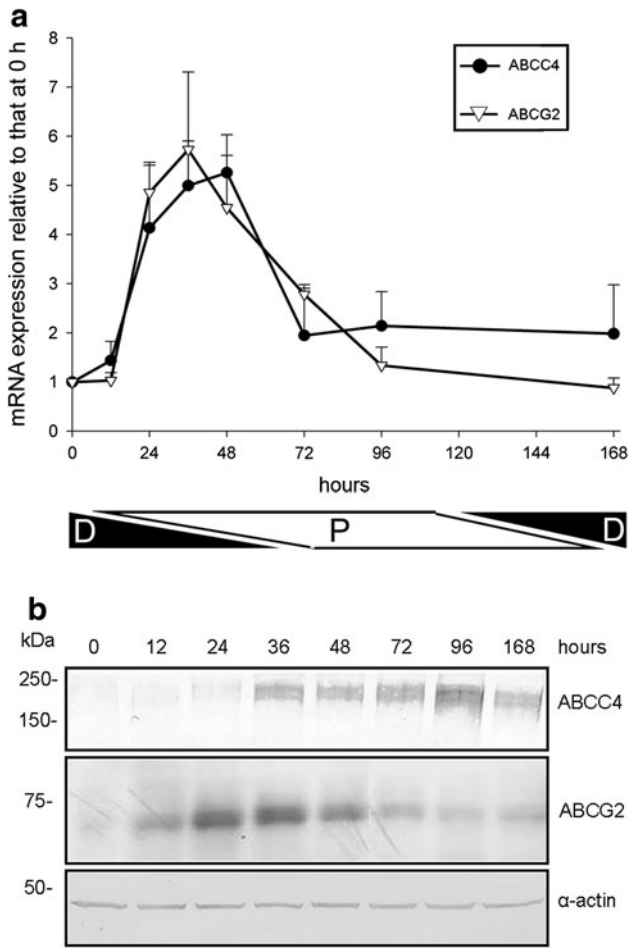

Fig. 1 Proliferation-related expressions of the ABCC4 and ABCG2 transporters in synchronized HaCaT cells. Changes in expression of the ABCC4 and ABCG2 genes were detected by real-time RT-PCR (**a**). Values (mean ± SEM) are shown relative to the expression of the 0-h sample at the end of the synchronization process; results were normalized to 18S rRNA. Expressions of the ABCC4 and ABCG2 proteins were detected by western blotting (**b**). Representative blots of three independent experiments are shown; α-actin was used as loading control. *D* differentiation phase, *P* proliferative phase

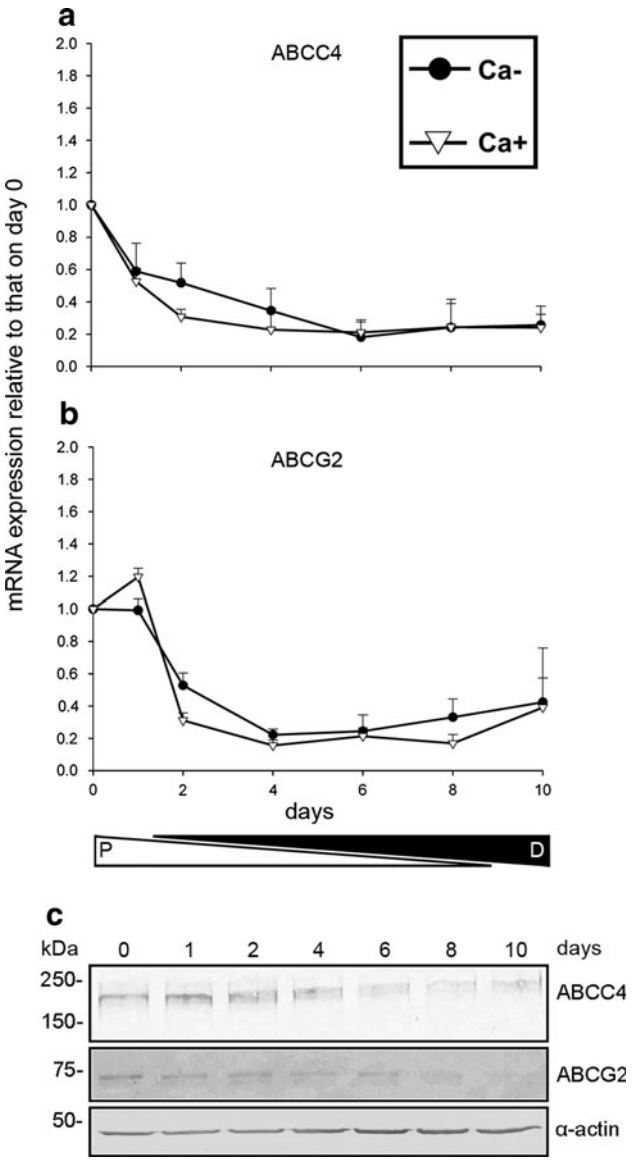

Fig. 2 Proliferation-related expressions of the ABCC4 and ABCG2 transporters during the in vitro differentiation of NHKs. Changes in expression of the ABCC4 (**a**) and ABCG2 (**b**) genes were detected by real-time RT-PCR. Values (mean ± SEM) are shown relative to the expression of the 0-day sample; results are normalized to 18S rRNA. Ca− control culture (0.09 mM Ca^{2+}), Ca+ high-Ca^{2+} culture (1.7 mM Ca^{2+}). Expressions of the ABCC4 and ABCG2 proteins were determined in low-Ca^{2+} containing cultures (Ca− control NHKs). Representative blots of three independent experiments are shown; α-actin was used as loading control (**c**). *P* proliferative phase, *D* differentiation phase

NHKs, the ABCC4 and ABCG2 transporters were elevated on day 0, but both proteins decreased along with keratinocyte differentiation and were almost undetectable by day 10. These alterations were similar under low- and high-Ca^{2+} conditions (Fig. 2c, only NHKs cultured in low-Ca^{2+} medium are shown). Immunocytochemical experiments confirmed the Western blot findings in both HaCaT cells and NHKs (Online Resources 5 and 6).

Functional analysis of ABCC4 and ABCG2 proteins in HaCaT cells and NHKs

Since we observed a proliferation-related expression for both ABCC4 and ABCG2 in HaCaT cells and NHKs, we next investigated whether these proteins are indispensable for keratinocyte proliferation. Gene-specific silencing and functional inhibition approaches were applied to HaCaT cells.

A marked decrease in the expression of the ABCC4 protein was observed in cells treated with siRNA specific to ABCC4 (siC4) relative to HaCaT cells treated with scrambled control siRNA (siK) (Fig. 3a). The silencing of

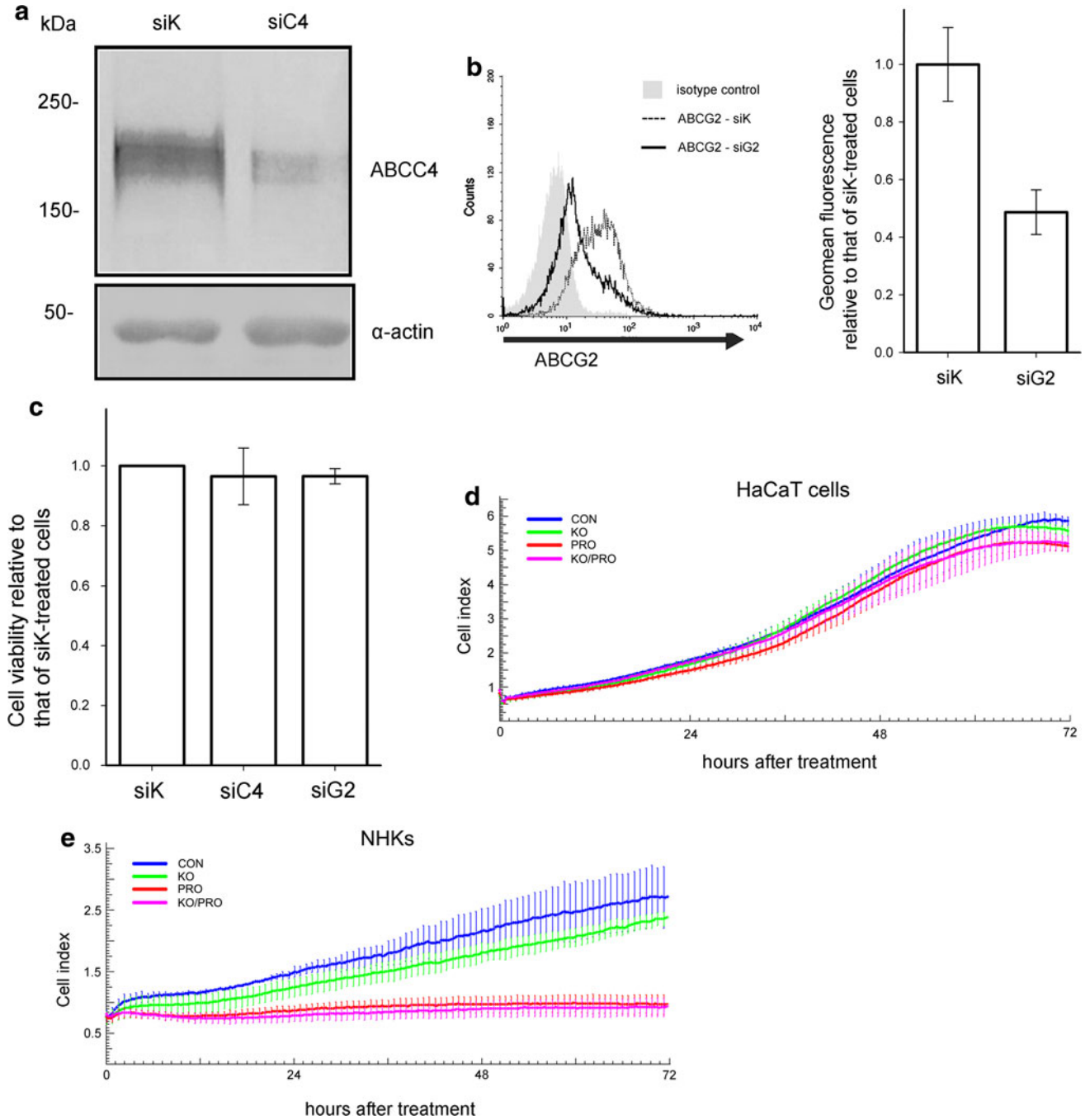

Fig. 3 Abrogation of ABCC4 and ABCG2 transporter functions in HaCaT cells. HaCaT keratinocytes were transfected with ABCC4- and ABCG2-specific siRNAs (siC4 and siG2, respectively) and a control scrambled siRNA (siK). The ABCC4 protein exhibited a marked reduction in siC4-transfected cells at 48 h following transfection. A representative blot is shown; α-actin was used as loading control (**a**). Cell surface ABCG2 protein was assessed by immunostaining and measured by flow cytometry 48 h after transfection. A representative histogram and the geometric mean ± SD relative to siK are shown (**b**). MTT cell viability assays were performed 72 h following ABCC4- and ABCG2-specific siRNA transfection (**c**). Real-time cell analysis of HaCaT cells (**d**) and NHKs (**e**) treated with the ABCG2-specific inhibitor Ko-134 (KO, 1 μM), or the ABCC4 inhibitor probenecid (PRO, 0.5 mM) or their combination (KO/PRO). Vehicle-treated cells (CON) were monitored as control. The length of the follow-up period was 72 h. Four parallel samples per experiment were measured and three independent experiments were performed

ABCG2 monitored by flow cytometry indicated that the ABCG2-specific siRNA (siG2) treatment decreased the level of the membrane ABCG2 protein to 48.7% relative to the cells treated with siK (Fig. 3b). The MTT assay did not reveal any difference in the viability of siC4-, siG2- and siK-transfected HaCaT cells (Fig. 3c).

In order to investigate the effects of functional inhibition of ABCC4 and ABCG2, we treated HaCaT keratinocytes with the inhibitors of ABCC4 and ABCG2 (0.5 mM probenecid and 1 μM Ko-134, respectively) for 72 h. For the assessment of cellular status, we applied a real-time non-invasive, impedance-based technique, the xCELLigence System. Disruption of the functions of ABCC4 alone, ABCG2 alone or both transporters simultaneously did not have any effect on the cell index (Fig. 3d). The function-inhibiting action of such concentrations of probenecid on ABCC-type transporters and that of Ko-134 on ABCG2 were demonstrated previously [1, 20].

These data indicated that neither gene silencing nor the functional inhibition of ABCC4 or ABCG2 affected the proliferation or the viability of HaCaT keratinocytes. However, because of the immortalized nature of the HaCaT cell line, it was necessary to confirm these findings on NHKs.

NHK were treated with Ko-134, a specific inhibitor of ABCG2, and probenecid, which inhibits ABCC-type transporters. The cellular status was monitored for 72 h with the xCELLigence System. There was no difference in cell index between the vehicle-treated and Ko-134-treated NHKs, but incubation with the ABCC inhibitor probenecid resulted in a significant decrease in the cell index (Fig. 3e).

In summary, the inhibition of ABCG2 did not affect cell proliferation in either HaCaT cells or NHKs. Disruption of the ABCC-type transporter function, however, resulted in a substantial decrease in the proliferation of NHKs, but not in that if HaCaT cells.

Discussion

Extensive research on transporters providing cancer cells with multidrug resistance has demonstrated that their role far exceeds merely chemoresistance. Although data are steadily accumulating on the physiological functions of xenobiotic transporters [11], only limited information is available on their roles in the epidermis.

Our systematic survey on eight xenobiotic transporter genes revealed that only the expressions of ABCC4 and ABCG2 were associated with keratinocyte proliferation in two keratinocyte model systems. Our results on the Ca^{2+}-induced differentiation of NHKs are generally in agreement with the observations of others, though we also noted some discrepancies. Kielar et al. [8] reported that the ABCC1, ABCC3 and ABCC4 genes were induced, while the ABCG2 gene was not regulated in differentiating keratinocytes. In contrast, our data indicated a notable downregulation of the ABCC4 and ABCG2 genes upon keratinocyte differentiation, whereas only minor changes in ABCC1 and ABCC3 gene expression were observed. We presume that the reason for these differences lies in the in vitro model systems used. Although we also applied elevated Ca^{2+} concentrations to initiate NHK differentiation, we introduced this high-Ca^{2+} medium to almost confluent cells, while Kielar et al. applied high-Ca^{2+} at the time of plating of the cells. Moreover, we observed that the ABCC4 and ABCG2 proteins are not expressed in highly differentiated HaCaT cells and NHKs, while high transporter protein levels are present in proliferating cells, supporting our gene expression data. Similar findings have been reported in other cell types as concerns the possible roles of both ABCC4 [7, 15] and ABCG2 [5, 10] in relation to cell proliferation.

We detected marked similarities in the transcriptional activities of the ABCC4 and ABCG2 genes. An earlier detailed promoter analysis on the ABCG2 gene demonstrated the presence of important positive and negative regulatory elements [2]. Recent studies identified several xenobiotic responsive promoter elements in both ABCC4 and ABCG2 promoters, controlled by the aryl hydrocarbon receptor, which plays an important role in stress-related signaling [19, 21].

Our functional studies suggested that neither ABCC4 nor ABCG2 is indispensable for the proliferation of HaCaT cells. Moreover, the specific inhibition of ABCG2 did not affect the NHKs proliferation either. ABCG2 has been shown to be involved in the cell cycle progression of tumor cell lines A549 and MCF7/MX [5], indicating differential functions of ABCG2 in normal and tumor cells. However, the ABCC-type transporter inhibitor probenecid exerted a deleterious effect on the proliferation of NHKs, which is in accordance with the results of Sassi et al. [15], showing that ABCC4 has a substantial role in cell proliferation through modulation of the level of secondary messenger cAMP. The immortalized nature of HaCaT cells may affect signaling pathways regulating cell proliferation, which could result in a decreased sensitivity to probenecid.

In summary, our systematic survey on the expression and function of xenobiotic transporters in HaCaT cells and NHKs indicated a similar proliferation-related regulation for ABCC4 and ABCG2 transporters. Our functional studies indicated that the inhibition of ABCC-type transporters by probenecid significantly affected NHK proliferation. Xenobiotic transporters could display substantial therapy-modulating effects in dermatological disorders. Detailed studies on their expression in NHKs and human epidermis may therefore promote an understanding of their functions and reveal new therapeutic approaches by targeting them with specific inhibitors.

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Conflict of interest The authors declare that they have no conflict of interest.

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