

Hallmarks of Atopic Skin Mimicked *In Vitro* by Means of a Skin Disease Model Based on *FLG* Knock-down

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Summary — Loss-of-function mutations in the filaggrin gene (*FLG*) are a strong predisposing factor for atopic dermatitis, although their relevance to the disease pathomechanism needs further elucidation. The generation of an *in vitro* model of atopic skin would not only permit further evaluation of the underlying pathogenetic mechanisms and the testing of new treatment options, but would also allow toxicological studies to be performed in a simple, rapid and inexpensive manner. In this study, we have knocked down *FLG* expression in human keratinocytes and created three-dimensional skin models, which we used to investigate the impact of *FLG* on epidermal maturation and on skin absorption and its response to irritation. Histopathological evaluation of the skin models showed impaired epidermal differentiation in the *FLG* knock-down model. In addition, skin irritation induced by an application of sodium dodecyl sulphate resulted in significantly higher lactate dehydrogenase leakage, and interleukin (IL)-6 and IL-8 levels, than in the control model. To assess the effect of filaggrin deficiency on skin absorption of topically applied agents, we quantified the percutaneous absorption of lipophilic and hydrophilic model drugs, finding clinical relevance only for lipophilic drugs. This study clearly demonstrates that important clinical characteristics of atopic skin can be mimicked by using *in vitro* skin models. The *FLG* knock-down construct is the first step toward an *in vitro* model that allows clinical and toxicological studies of atopic-like skin.

Key words: atopic dermatitis, filaggrin, *in vitro* skin disease model, siRNA, skin barrier function.

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Introduction

Atopic dermatitis (AD) is a major public health problem of the Western world. Currently, about 10–20% of children and 1–3% of adults are affected, but its prevalence is increasing. AD exhibits a multifactorial disease pattern, the pathophysiology of which is not yet fully understood (1, 2). To date, it is known that gene mutations, such as loss-of-function mutations in the filaggrin gene (*FLG*), the individual's environment, and immunological factors, such as increased levels of T helper 2 (Th2) cytokines initiated by, for example, interleukin (IL)-1  , IL-6 or thymic stromal lymphopoietin (TSLP), considerably contribute to the pathogenesis (3–5).

An intact epidermal structure is a prerequisite for the skin to function adequately as a physical and chemical barrier. In AD, this skin barrier function is disturbed. Changes in skin ceramides and the altered expression of enzymes involved in the development of epidermal adhesion structures contribute to the breakdown of the epidermal barrier in AD patients (1). The impact of a mutation in

FLG on the pathogenesis of AD has been highlighted — loss-of-function mutations in *FLG* are a strong predisposing factor for AD, and are the most-widely occurring genetic risk factor for AD known to date (6). Such mutations have been identified in up to 50% of all patients (7, 8). In addition, mutations in *FLG* not only predispose to AD, but can also cause ichthyosis vulgaris (9, 10). *FLG* loss-of-function mutations are known to provoke intercellular barrier abnormality, reducing the skin's inflammatory threshold to topical irritants and antigens, which triggers inflammatory processes (10–12).

Despite the high prevalence of AD, valid data on the absorption of drugs/xenobiotics in diseased skin are lacking. Most likely, absorption in diseased skin differs from that of healthy skin due to the altered barrier function, possibly resulting in the higher systemic availability of, for example, topically applied drugs or environmental compounds. To improve the understanding of AD and the development of new treatment options, and for hazard analysis, non-clinical studies have to

become possible. Various animal models are described in the literature, including the flaky tail mouse that has a *FLG* loss-of-function mutation (11), but problems such as the limited availability, limited reproducibility and interspecies transferability of the results and ethical issues remain unsolved (13, 14). Various attempts have been made to mimic AD skin barrier disruption in human skin *ex vivo* (15), but always with a poor outcome.

Over recent decades, tissue engineering has taken a great step forward — *in vitro* models have furthered the understanding of epidermal cell biology and now provide novel experimental systems for pharmacological, toxicological and cosmetic studies (16). Various *in vitro* skin models mimic normal human skin, and some have already been validated for toxicity testing (17–19). In contrast, the development of *in vitro* skin disease models is still in its infancy. Through gene silencing, or by co-culturing normal skin cells and cells obtained from biopsies of lesional skin, disease models of squamous cell carcinoma (20) and peeling skin disease (21) have been generated. Nevertheless, an *in vitro* model for AD, one of the most common skin diseases in industrialised countries, is still elusive. One reason might be the complexity of its pathogenesis. An *in vitro* model for filaggrin deficiency has been described recently (22), but a detailed analysis of the skin barrier function, which is crucial for its use as an AD model, was not performed.

Therefore, in this study, we knocked down *FLG* in normal human keratinocytes (NHKs) in order to reconstruct diseased full-thickness human skin and to be able to address the following questions: 1) does *FLG* knock-down affect maturation of the epidermal barrier?; 2) does the absence of *FLG* affect the response of skin models to local irritants?; and 3) does the knock-down of *FLG* have an impact on skin absorption of xenobiotics?

Materials and Methods

Cell culture and siRNA transfection

NHKs and fibroblasts were isolated from juvenile foreskin taken during circumcision surgery (with ethical consent), and were cultured as described previously (23, 24). NHKs were transfected with *FLG*-specific siRNA (sequence 5'-CAGCUCCAGACAAUCAGGCACUCAU-3'; NM_002016, Invitrogen, Darmstadt, Germany), or non-coding small interfering RNA (siRNA; sequence not shared by Invitrogen) as a control, by using HiPerFect® transfection reagent (Qiagen, Hilden, Germany) and following the manufacturer's instructions. Prior to transfection, siRNA was incubated with 3 µl of HiPerFect transfection reagent in 500 µl of keratinocyte growth medium (KGM; Lonza, Cologne, Germany).

Preparation of normal and diseased *in vitro* skin models

In vitro skin models were generated as described previously (25). Briefly, fibroblasts (2.5×10^5 /well), fetal calf serum (FCS; 0.3 ml/well) and bovine collagen I (Nutacon, Leimuiden, The Netherlands) were brought to neutral pH and decanted (total volume per well 2.5 ml) into three-dimensional (3-D) culture filter inserts for 6-well plates with a growth area of 4.2 cm² (BD Biosciences, Heidelberg, Germany). After incubation for 3 hours at 37°C, 2 ml KGM were added and the system was transferred to an incubator with 5% (v/v) CO₂ and 95% (v/v) humidity. After a second incubation, for 2 hours, NHKs in KGM medium (4.2×10^6 /well, with or without the *FLG* knock-down) were added on top of the collagen matrix and post-incubated for 24 hours at 37°C and 5% CO₂. The following day, the constructs were lifted to the air-liquid interface and the medium was changed to a proprietary differentiation medium (25). The cell models were cultured for 14 days (at 37°C, with 5% CO₂ and 95% humidity), with a change of medium every second day.

Evaluation of the knock-down efficiency

The efficiency of the *FLG* knock-down in the skin models was determined for every batch. At day 14, the constructs were harvested, and 10 mm discs were punched by using a biopsy punch. The tissues were frozen immediately with liquid nitrogen, and then milled for 30 seconds at 25 Hz by using a TissueLyzer (Qiagen). Subsequently, RNA was isolated with NucleoSpin® RNA II, according to the manufacturer's instructions. For cDNA synthesis, the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas GmbH, St Leon Roth, Germany) was used, and synthesis was carried out according to the manufacturer's instructions.

For the relative quantification of *FLG* expression, RT-PCR was performed by using the SYBR Green I MasterPLUS kit (Roche, Penzberg, Germany) according to the manufacturer's protocol. The *FLG* primer sequences were: forward 5'-AAGGAACCTTCTGGAAAAGGAATTTTC-3', reverse 5'-TTGTGGTCTATATCCAAGTGATCCAT-3'. Two housekeeping genes, *YWHAZ* and *GAPDH*, served as controls.

HE-staining and immunostaining analysis of skin differentiation markers

After culture for 14 days, the skin models were fixed in 4% (v/v) formalin solution, dehydrated and then embedded in paraffin. Serial 5 µm sections were cut by using a microtome and stained

with haematoxylin-eosin (HE) by following the conventional staining procedure. Additionally, a number of skin differentiation markers were detected by immunostaining (Table 1). For collagen IV, cytokeratin 14, cytokeratin 10 and involucrin, the DAKO ChemMate™ Detection Kit (Alkaline Phosphatase/RED, Rabbit/Mouse; DAKO, Eching, Germany) was used. For filaggrin, immunostaining with fluorescence-labelled antibodies, according to routine histological protocols, was performed. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

Skin irritation testing

Skin irritation testing and data evaluation were performed on the basis of the slightly adjusted standard operating procedure (SOP) of the ECVAM Skin Irritation Validation Study (18). Briefly, 10 µl of a sodium dodecyl sulphate (SDS) solution (1% [w/v] in PBS) were applied directly onto the normal and *FLG* knock-down skin models. PBS-treated skin models served as controls. After a 15-minute incubation at 37°C, the test solutions were carefully rinsed off with PBS, and the skin constructs were post-incubated for 24 hours at 37°C. Subsequently, data on cell viability were obtained by using the MTT assay.

Enzyme-linked immunosorbent assay (ELISA)

The release of the irritation markers IL-6 and IL-8, was determined by means of an ELISA. Twenty-four hours after exposure to SDS, the cell medium was removed and stored at -80°C until required for further use. The cytokines were quantified with IL-6/IL-8 DuoSet (R&D Systems, Wiesbaden, Germany), by following the manufacturer's instructions (26, 27).

Lactate dehydrogenase leakage

After the 24-hour post-incubation, 300 µl of cell medium were removed and analysed for the presence of lactate dehydrogenase (LDH) with the Lactate Dehydrogenase Cytotoxicity Detection Kit, according to the International Federation of Clinical Chemistry method, Version 2 (Roche; 28). When the cell membrane is damaged, the cells release LDH, which converts a tetrazolium salt added to the culture medium into formazan. Formazan levels in the supernatant were assessed by measuring absorbance at 490 nm with a COBAS INTEGRA® 400 plus spectrophotometer (Roche).

Cell viability

Cell viability was assessed by using the MTT test, according to a validated test protocol (18). By measuring changes in absorbance readings, as a result of formazan accumulation in the culture medium, cell numbers can be estimated. PBS-treated skin constructs served as the reference; their absorbance values were set to 100%. The experiment was repeated with at least three different batches per skin model.

Skin absorption testing

For skin absorption testing, stock solutions of testosterone (40 µg/ml, 2% [v/v] Igepal® CA-630) and caffeine (1000 µg/ml; all from Sigma-Aldrich, Munich, Germany) were used. The stock solutions were spiked with an appropriate amount of the radiolabelled compound, to a total radioactivity of 2 µCi/ml. Solutions were stored at 4°C and remained stable for at least 4 weeks (29).

By using the static setup and the infinite dose approach, permeation tests (Franz-type diffusion cells, diameter 15 mm, volume 12 ml; Permeaguard, Bethlehem, PA, USA) were performed in triplicate.

Table 1: List of the antibodies used for immunostaining

Antigen	Species	Company	Dilution
Filaggrin	Rabbit	SantaCruz ^a	1:100
Collagen IV	Mouse	Dako ^b	1:50
Cytokeratin 10	Mouse	BioGenex ^c	1:40
Cytokeratin 14	Mouse	BioGenex ^c	1:50
Involucrin	Rabbit	Biomedical Technologies Inc. ^d	1:10–1:100
Anti-rabbit Alexa 488	Goat	Invitrogen ^e	1:400
Anti-rabbit/mouse alkaline phosphatase	Goat	Dako ^b	Undiluted

^aSanta Cruz, CA, USA; ^bGlostrup, Denmark; ^cSan Ramon, CA, USA; ^dStoughton, MA, USA and ^eInvitrogen, Darmstadt, Germany.

cate, according to validated test procedures (19, 30).

Statistical analysis

The data were analysed by using ANOVA and the paired *t*-test, and $p \leq 0.05$ was defined as the level of significance.

Results

FLG knock-down results in disturbed development and maturation of the epidermal barrier

After 14 days in culture, the efficiency of the *FLG* knock-down in the skin model was determined by using RT-PCR analysis and was found to be $82.2 \pm 10.7\%$. To ensure the specific knock-down of *FLG*, 3-D models derived from NHKs that had been transfected with non-coding siRNA were also generated. These produced $87 \pm 20.4\%$ of the *FLG* mRNA levels present in the skin models made from non-transfected cells (Figure 1a). Furthermore, *FLG* synthesis was determined by immunofluorescence staining, which revealed strong *FLG* staining in the normal skin model, but considerably reduced fluorescence in the knock-down model (Figure 2).

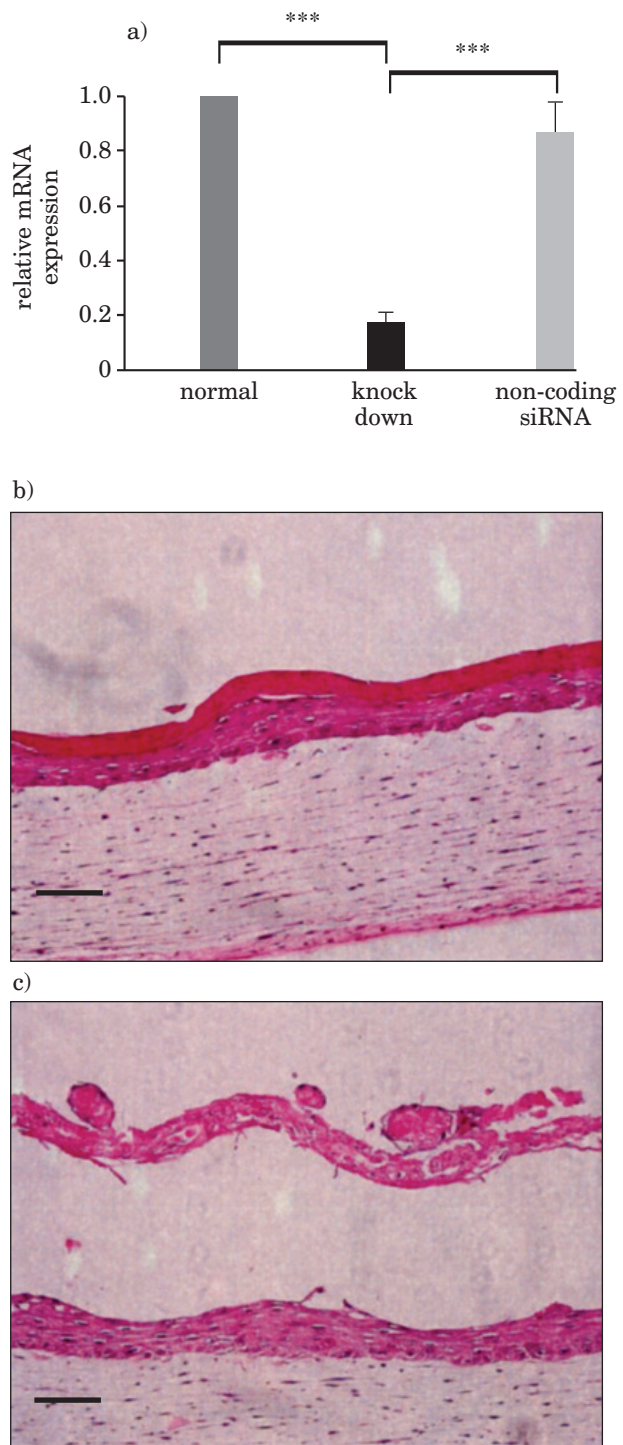
HE staining revealed that *FLG* knock-down resulted in obvious and strong alterations in the morphology of the viable epidermis and stratum corneum. In the normal skin model, all the epidermal layers were clearly structured, and the typical flattening of the keratinocytes that occurs during epidermal differentiation could be seen (Figure 1b). By contrast, transfected (*FLG* knock-down) skin constructs exhibited an obviously disturbed development of the stratum corneum (Figure 1c), with intercellular and intracellular spongiosis as a permanent feature. The typical flattening of the keratinocytes and the loss of nuclei during keratinocyte differentiation were less pronounced in the skin disease model. Furthermore, the staining clearly revealed typically erected cells and their compact organisation in the basal epidermal layer of the normal tissue (Figure 1b). This was not observed in the *FLG* knock-down model (Figure 1c).

The epidermal differentiation markers, involucrin, keratin 10, keratin 14 and collagen IV, were expressed in the skin constructs, both normal and diseased, but with varying levels of intensity (data not shown).

FLG deficiency increases skin sensitivity to topically applied irritants

To assess the influence of *FLG* on the barrier properties of human skin, in terms of its ability

Figure 1: Histological evaluation of skin models and determination of the knock-down efficiency

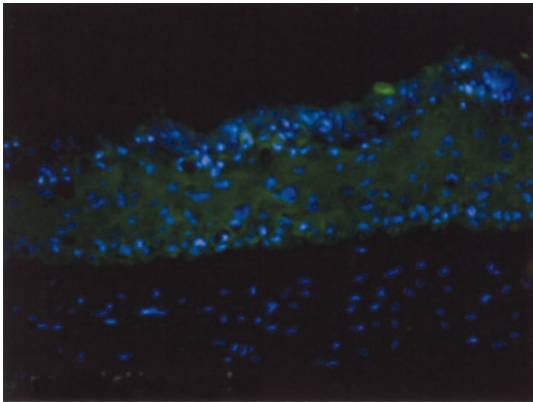


a) Skin constructs grown from NHKs transfected with *FLG*-specific siRNA (black bar) show efficient knock-down of *FLG* expression by 82% ($n = 9$, $***p < 0.0001$). No knock-down was observed when exposing NHK to non-coding siRNA (grey bar) (3 batches). Representative histological images of b) a normal construct and c) a construct with *FLG* knock-down cultured for 14 days (10× magnification).

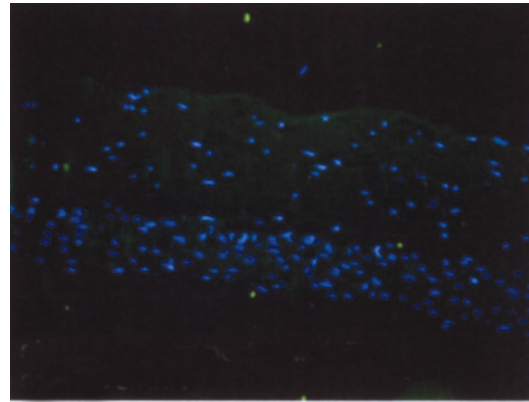
Scale bars = 100µm.

Figure 2: Immunofluorescence of FLG in a normal skin construct and in a skin construct made from keratinocytes with FLG knock-down

Normal skin model



Skin model transfected with FLG-specific siRNA



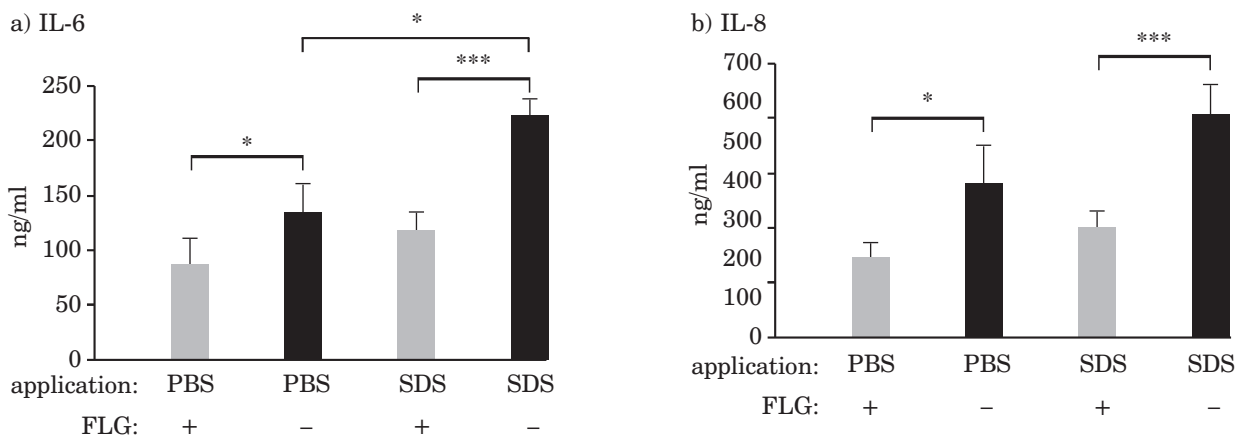
Strong immunofluorescence, indicative of FLG, is visible in the normal skin construct (left image) while considerably reduced FLG synthesis is present in skin constructs grown from siRNA-treated keratinocytes (right image). The nuclei were stained with DAPI (blue fluorescence; 20× magnification).

to protect against local irritants, the standard irritant, SDS, was applied topically onto normal and *FLG* knock-down skin models.

For the specific assessment of skin irritation, the release of cytokines IL-6 and IL-8 was determined following exposure to PBS and SDS. In general, IL-8 release (200–500 ng/ml) significantly exceeded

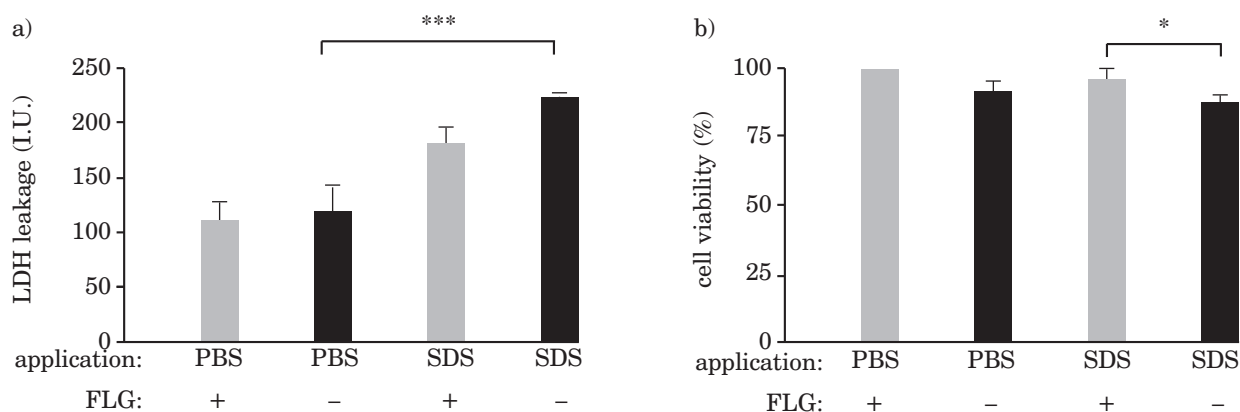
IL-6 release (80–200 ng/ml). IL-6 and IL-8 levels were significantly higher in the transfected skin model compared to the control model, and cytokine release after SDS application was particularly increased in the transfected skin model. However, even PBS was irritating for the disease model (Figure 3a and b).

Figure 3: The effects of SDS on IL-6 and IL-8 release



IL-6 and IL-8 release following the application of SDS was higher in the transfected skin model, compared to the normal skin model; PBS served as the negative control (3 batches, mean \pm SEM, * p < 0.05 and *** p < 0.001).

■ = normal skin model (+FLG); ■ = FLG knock-down skin model (-FLG).

Figure 4: The effects of SDS on LDH leakage and cell viability

a) LDH leakage following application of SDS. LDH release was higher in the knock-down construct compared to the normal model (5 batches, mean \pm SD; *** $p < 0.001$). b) Cell viability of normal skin model and FLG knock-down model after treatment with the control PBS or the irritant SDS (3 batches, mean \pm SD; * $p < 0.05$).

■ = normal skin model (+FLG); ■ = FLG knock-down skin model (-FLG)

The application of SDS also caused a significant increase in LDH release in both skin models, but tissue damage was most pronounced in the FLG-deficient constructs (Figure 4a). Again, even the application of PBS resulted in slightly higher LDH release in the siRNA-transfected skin model compared to the normal tissue.

In addition, cell viability was assessed by using the MTT assay. After treatment of the normal skin model with SDS, mean cell viability remained almost constant — $96 \pm 0.07\%$ (with exposure to PBS as the 100% reference point). In the FLG knock-down model, significantly lower cell viabilities were measured: PBS-treated tissues = $92 \pm 0.06\%$, SDS-treated tissues = $87 \pm 0.05\%$ (Figure 4b).

FLG knock-down results in altered skin absorption

To determine the impact of FLG on the percutaneous absorption of drugs/xenobiotics, skin absorption studies were performed according to Organisation for Economic Co-operation and Development (OECD) guidelines and validated test procedures (19). Testosterone ($\log P = 3.47$) served as a model substance for lipophilic compounds, and caffeine ($\log P = -0.08$) was used as a model substance for hydrophilic compounds. In the case of testosterone, the FLG knock-down model showed higher permeability (Figure 5b) than the normal skin model. Mean apparent permeability coefficient (P_{app}) values \pm SEM were $9.53 \times 10^{-6} \pm 1.81 \text{ cm/s}$ for the FLG-deficient model, and $6.32 \times$

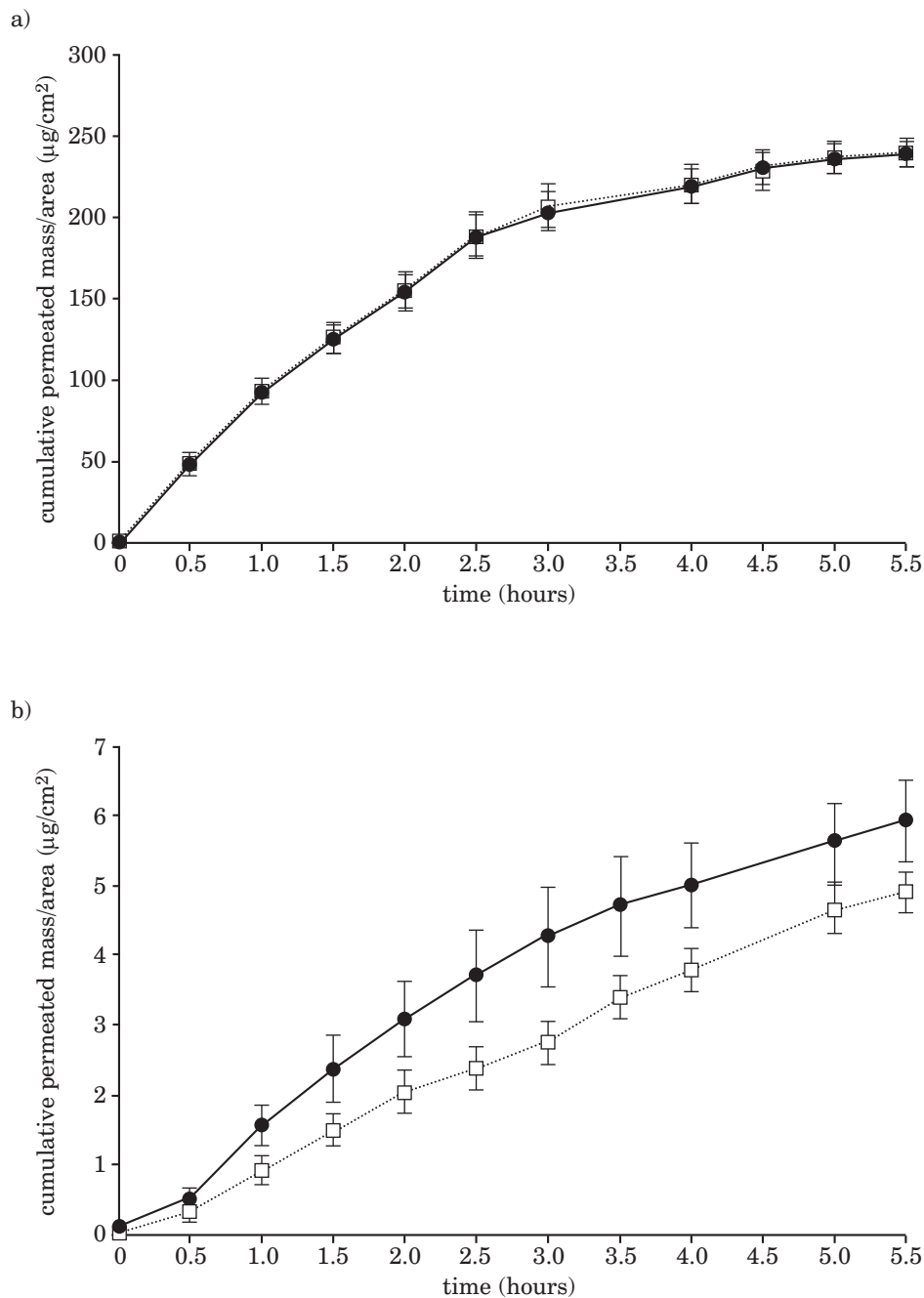
$10^{-6} \pm 0.83 \text{ cm/s}$ for the normal skin model. The lag-times (mean values \pm SEM) of testosterone did not differ, with -0.15 ± 0.07 hours for the FLG-deficient skin model and -0.10 ± 0.10 hours for the normal skin model. Caffeine permeability in the FLG knock-down model was only slightly enhanced compared to that in the normal skin model (Figure 5a). Mean P_{app} values \pm SEM were $19.58 \times 10^{-6} \pm 1.07 \text{ cm/s}$ (lag-time -0.17 ± 0.05 hours) for the knock-down skin model, and $18.35 \times 10^{-6} \pm 1.18 \text{ cm/s}$ (lag time -0.44 ± 0.12 hours) for the normal skin model.

Discussion

FLG loss-of-function mutations occur in up to 50% of AD patients tested, and were recognised as a major predisposing factor for this chronic inflammatory skin disease (6, 31, 32). Although further elucidation is required to unravel the impact of FLG deficiency on the pathogenesis and maintenance of AD, we based our *in vitro* skin disease model on this frequent genetic defect. We clearly demonstrated that FLG-deficient skin constructs can mimic hallmarks of AD *in vitro*, namely, disturbed epidermal maturation, impaired barrier function and higher susceptibility to irritants.

We have shown that FLG knock-down models exhibit histological features that are also found in skin biopsies of AD patients (33). In contrast to our results, a previous study had failed to find any influence of FLG knock-down on keratinocyte differentiation and stratum corneum development (22), differences that are most likely due to the

Figure 5: Skin permeation of caffeine and testosterone in the *FLG* knock-down model and the normal skin model



Cumulative amounts (mean values \pm SEM, 3 batches) of a) permeated caffeine and b) testosterone in the *FLG* knock-down model ($-FLG$) and the normal skin model ($+FLG$) plotted against time.

□ = $+FLG$; ● = $-FLG$.

shorter cultivation period of seven days (compared to 14 days for our model). Apparently, the influence of *FLG* deficiency on epidermal maturation does not become visible until cultivation periods exceed one week in duration. Mildner *et al.* (22)

also described the enhanced UV sensitivity of skin models due to *FLG* deficiency, and the facilitated skin absorption of the fluorescent dye, Lucifer yellow. Our study provides complementary data. We have demonstrated the impact of *FLG* knock-down

on the skin absorption of caffeine and testosterone (OECD standardised test substances) and have shown, by determining the release of the pro-inflammatory cytokines IL-6 and IL-8, that *FLG* knock-down models have an increased susceptibility to skin irritation.

Our findings substantiate the idea that *FLG* deficiency causes disturbances of skin barrier development, which contribute to, and result in, the histological characteristics of AD patients, i.e. skin lesions. Nevertheless, it has to be mentioned that 50% of AD patients tested do not have mutations in *FLG*, meaning that inherited abnormalities in other proteins, such as other skin barrier components, or barrier processing enzymes such as serine proteases or transglutaminases, also contribute to barrier disruption (7, 31).

Lately, there has been a paradigm shift regarding the pathogenesis of AD. Over several decades, it was believed that primary abnormalities of the immune system led to the development of AD (the historical 'inside–outside' view). During the last 3–5 years, evidence has emerged that AD pathogenesis results from inherited and acquired skin barrier deficiencies (e.g. *FLG* deficiency), leading to an activation of the immune response which further contributes to skin barrier damage ('outside–inside–outside' mechanism [31]). Thus, we investigated whether it would be possible to mimic the initial mechanisms of skin sensitisation *in vitro*, and determined the impact of *FLG* deficiency on skin irritation. We found significantly higher release of LDH (Figure 4a) and of the pro-inflammatory cytokines, IL-6 and IL-8 (Figure 3), and decreased cell viability (Figure 4b) in the *FLG* knock-down models. Furthermore, in all three approaches, the application of PBS onto the *FLG*-deficient models caused irritation. This is well in accordance with the *in vivo* situation, as water is a well-known irritant for AD patients (34). It was not possible to assess IL-1 α levels, since this cytokine interferes considerably with the fibroblasts of the dermis equivalent and thus cannot be used as a skin irritation marker in full-thickness skin models (18). Nevertheless, initial signs of skin inflammation were found: it is known that IL-6, in synergy with IL-1 α , controls the initial step in peripheral T-cell activation and stimulates the further release of pro-inflammatory cytokines (35), resulting in the Th2-dominated immune response that is a hallmark of this chronic skin disease (36). IL-8 is important for the recruitment of immunocompetent cells into the skin (37). For the sake of completeness, it has to be mentioned that other cytokines, such as thymic stromal lymphopoietin (TSLP), also promote a Th2 response (5).

Topical treatment of skin diseases is the favoured option, in order to avoid systemic side-effects and to increase patient compliance. Although AD is a major public health problem, almost nothing is

known about the absorption characteristics of atopic skin. *In vivo* studies are problematic due to ethical issues, as most AD patients are babies or toddlers, and *in vitro* test systems are currently lacking. Thus, we investigated the dermal absorption of testosterone (OECD standard lipophilic drug) and caffeine (OECD standard hydrophilic drug; 19) in our *in vitro* skin models. For testosterone, clear differences in skin absorption between the normal and *FLG* knock-down models were observed — skin absorption was higher in the disease model (Figure 5b). Surprisingly, only a slight difference was seen for the hydrophilic agent, caffeine (Figure 5a). These results indicate that *FLG* deficiency increases, in particular, the dermal absorption of lipophilic compounds. These findings are well in accordance with clinical experience (38), and are of great relevance for the therapy of AD patients. Glucocorticoids (GCs) are still the most important drugs for the topical treatment of AD exacerbations, but their systemic bioavailability might be underestimated. In contrast, for the hydrophilic calcineurin inhibitors, absorption-related systemic effects seem to be less relevant, but concerns about malignancy must then be taken into account. The impairment of the penetration barrier is clearly due to the lack of *FLG*, which is mandatory for the correct formation of the cornified envelope, and thus, for the formation of an intact epidermal barrier.

As was shown here, disease-related symptoms and reactions can be mimicked by using *in vitro* models. As a result, carrying out preclinical studies, 'proof-of-concept' testing and the risk assessment of new drugs or therapeutic approaches, could become feasible in a relatively inexpensive and simple manner.

Our *FLG* knock-down model does not reflect, in full, the multifactorial disease pattern of AD, but advanced models could be developed, with modification of the expression of other structural proteins (e.g. involucrin, etc.) or enzymes (e.g. serine proteases, transglutaminases), which play a role in AD. Furthermore, the inflammatory component may be added to the skin model by implementation of Th2 cells or dendritic cells.

Conclusion

We have shown that important clinical characteristics of atopic skin can be mimicked by using *FLG*-deficient *in vitro* skin models. We demonstrated that *FLG* deficiency disturbs epidermal maturation and skin barrier development, which results in enhanced susceptibility to skin irritation. These findings substantiate the hypothesis of an outside–inside–outside pathogenesis mechanism for AD. Furthermore, the suitability of this *in vitro* model of *FLG*-deficient skin as a test system for, for example, skin absorption or risk assessment

studies, has been demonstrated. The *FLG* knock-down model may form a basis for future development of a more complex *in vitro* skin disease model of AD.

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