



Performance of the N/TERT epidermal model for skin sensitizer identification via Nrf2-Keap1-ARE pathway activation



Mariam Alloul-Ramdhani, Cornelis P. Tensen, Abdoelwaheb El Ghalbzouri *

Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands

ARTICLE INFO

Article history:

Received 30 July 2013

Accepted 9 April 2014

Available online 1 May 2014

Keywords:

N/TERT

Reconstructed human epidermis

Skin sensitization

Keap1

Nrf2

DNCB

ABSTRACT

Animal testing of chemical ingredients for cosmetic purposes is prohibited. Therefore there is an urgent need for *in vitro* models to identify chemical allergens. In human skin, keratinocytes (KCs) are abundantly present and are key players in initiation of allergic contact dermatitis. One of the pathways that has been shown to be induced by sensitizers is the Keap1-Nrf2-ARE pathway. In this study we compared the response of four keratinocyte-based models including (a) primary human KCs, (b) N/TERT monolayer cultures, (c) the Leiden Epidermal models (LEMs) and (d) the N/TERT epidermal models (NEMs). All keratinocyte-based models were subjected to chemical exposure of the sensitizer 2,4-dinitrochlorobenzene (DNCB) and irritant Sodium dodecyl sulfate (SDS) at nontoxic concentrations. Activation of the Keap1-Nrf2-ARE pathway was evaluated by measuring Nrf2 protein levels as well as nuclear translocation and activation of transcriptional targets of Nrf2. Results show that the Keap1-Nrf2-ARE pathway is activated by the sensitizer DNCB in monolayer keratinocytes and as well as the LEMs and NEMs and not by the irritant SDS. Collectively our data demonstrate that the N/TERT models respond similarly as primary KCs and could therefore serve as an alternative model for skin sensitizer identification, thereby overcoming the need for primary skin tissue.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Human skin is the first barrier against chemical insults and pathogens and consists for 90% of keratinocytes (KCs). Several lines of evidence support the critical role KCs have as key players in the early initiation of Allergic contact dermatitis (ACD) (Kaplan et al., 2012; Nestle et al., 2009; Nickoloff, 2006; Kollisch et al., 2005). ACD is a common occupational health problem, which results in a skin T-cell mediated inflammation caused by chemical sensitizers that make contact with the skin. The first phase of ACD is referred to as the sensitization phase, which does not lead to clinical manifestations. During this phase chemical sensitizers act as haptens as they have the ability to bind skin-resident carrier proteins. Haptens can efficiently penetrate through the skin and make covalent bindings with carrier proteins. This hapten-protein complex is recognized by the innate immune system leading to production of pro-inflammatory mediators. As a consequence, effector T cells are mediated and enter the blood circulation. The second phase (elicitation) takes place after re-exposure of the skin to the hapten inducing inflammation (Kimber and Dearman, 2002;

Cavani, 2008; Gober and Gaspari, 2008; Vocanson et al., 2009; Kaplan et al., 2012).

In order to guarantee the safety of compounds used in the chemical and cosmetic industry, it is essential to identify and predict the sensitizing capacity of these ingredients. At the moment, animal tests such as the local lymph node assay (LLNA) (Kimber et al., 1994; Basketter et al., 2007) are used to identify chemical sensitizers and to classify them according to their potency. Reducing, replacing and refinement of the use of animal experimentation in the cosmetic industry has led to the introduction of the EU 7th Amendment to the cosmetic directive and the implementation of the Registration Evaluation Authorization and Restriction of Chemicals (REACH) regulation. Although there are many promising tests in development, to date there are no *in vitro* test models yet validated by the European Centre for the Validation of Alternative methods (ECVAM). Therefore there is an increasing need for reliable *in vitro* models that are able to identify chemicals causing skin sensitization.

A significant body of data has emerged suggesting that the oxidative stress pathway is a key pathway induced by sensitizers. Sensor protein Keap1 (Kelch-like ECH-associated protein 1) is present as a complex with Nrf2 leading to its constitutive proteasomal degradation. Covalent binding of chemical allergens to Keap1 through activation of the reactive cysteine residues of Keap1, results into

* Corresponding author. Address: Department of Dermatology, Einthovenweg 20, 2333 ZC, Leiden, The Netherlands. Tel.: +31 71 526 9365.

E-mail address: a.ghalbzouri@lumc.nl (A. El Ghalbzouri).

dissociation of the protein complex and stabilization of Nrf2 levels. Subsequently, Nrf2 translocates to the nucleus and induces transcription of genes containing an antioxidant response element (ARE) (Natsch and Emter, 2008; Natsch 2010; Vandebriel and van Loveren, 2010; Kaplan et al., 2012).

Several human cell lines have been developed for skin sensitizer prediction (e.g. MUTZ-3, THP-1, NCTC2455) either at transcription level or cytokine level (Ade et al., 2009; Saito et al., 2013a,b; Corsini et al., 2009). Here we focus on the KCs as they are the main building blocks of the skin and because they have an important role in initiation of ACD. The KC cell line HaCat has been used for the development of several *in vitro* skin sensitization identification tests (Vandebriel and van Loveren, 2010; Emter et al., 2010; McKim et al., 2010, van der Veen et al., 2013). In addition, several commercial available epidermal models such as EpiCS[®] (CellSystems, Germany), Epiderm[™] (MatTek, USA) and SkinEthic[™] RHE (L'Oreal, France), have been used for skin sensitization identification by measuring IL-18 secretion or transcription activation (Gibbs et al., 2013a,b; McKim et al., 2012; Saito et al., 2013a,b). In these models, sensitizers mainly induced transcription activation of the Nrf2 dependent genes.

The use of human based reconstructed skin models for immune-toxic screening has major advantages compared to conventional monolayer cell lines since it consists of a competent barrier. This allows topical application of chemicals with low aqueous solubility and finished (cosmetic) products. In order to apply and evaluate the penetration of a drug, compound or chemical through the skin, such *in vitro* models should recapitulate a competent skin barrier (Ponec et al., 1997; El Ghalbzouri et al., 2008; Thakoersing et al., 2010; Thakoersing et al. 2012). More important is the xenobiotic metabolizing capacity of these *in vitro* skin models as skin penetration is considered to be the key route for inducing sensitization (Hu et al., 2010; Neis et al., 2010; Gotz et al., 2012; Hewitt et al., 2013). Currently, most epidermal models are engineered using primary KCs in order to mimic *in vivo* characteristics (Ponec et al., 1997; El Ghalbzouri et al., 2008; Thakoersing et al., 2010). However, the use of a KC cell line that generates a reconstructed epidermis is desired and considered as an added value since the bio-variability (donor-to-donor variation) is drastically reduced and offers a constant supply of skin tissue. Here we demonstrate that the N/TERT epidermal model (NEM) shows similar activation of the Keap1-Nrf2-ARE pathway as the Leiden epidermal model (LEM) that is generated with primary KCs. This indicates that the NEM is a promising screening tool to predict the sensitizing potential of chemicals and may contribute to the reduction of animal experimentation.

2. Materials and methods

2.1. Cell culture

2.1.1. Primary human KCs

Primary human KCs were isolated from human mammary skin after informed consent according to the principles and guidelines of the Declaration of Helsinki, as described earlier (Ponec et al., 1997; El Ghalbzouri et al., 2008). In short, fresh skin tissue was obtained from surgical mammary reductions. First the epidermis was enzymatically separated from the dermis overnight using dispase II (Roche Diagnostics, Mannheim, Germany). The next day KCs were isolated from the epidermis using trypsin for 15 min at 37 °C followed by trypsin inactivation. Cells were filtered using a 70 µm cell strainer (BD Biosciences, Breda, The Netherlands) and cultured in keratinocyte medium at 37 °C and 7.3% CO₂. Primary KCs were cultured in DMEM/Ham's F12 medium (ratio 3:1) supplemented with 5% HyClone calf serum (Greiner, Nürtingen,

Germany), 0.5 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Breda, the Netherlands). In all experiments secondary cultures were used. Cultures were refreshed every 2 days.

2.1.2. N/TERT keratinocyte cell line

Human N/TERT cell line (J. Rheinwald laboratory, Harvard Medical School, Boston, USA) is an hTERT-immortalized human keratinocyte cell line (Dickson et al., 2000). Cells were cultured at 37 °C and 7.3% CO₂ in keratinocyte-serum free medium (K-SFM) supplemented with the following final concentrations; 25 µg/ml Bovine pituitary extract (BPE), 0.4 mM CaCl₂, 0.2 ng/ml hEGF, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Breda, the Netherlands). Cultures were refreshed every 2 days.

2.1.3. Reconstructed epidermis

The LEMs and the NEMs were constructed by seeding 200,000 cells on insert filters (12 wells plate, polyester membrane, Costar) in Dermalife K medium including life-factors (Lifeline Cell Technology, Walkersville, MD) until full density was reached. Thereafter cells were cultured in CnT-BM.3-500 medium (basal medium plus supplement kit (CnT-02-3DP.S), CellnTec, Bern, Switzerland). This medium was supplemented with 2.4×10^{-5} M bovine serum albumin, 25 µM palmitic acid, 15 µM linoleic acid and 7 µM arachidonic acid (Sigma). Next, cultures were lifted to air/liquid interface and after 1 day the linoleic acid concentration was increased to 30 µM. Medium was refreshed every 2 days. LEMs and NEMs were air-exposed for 12 days at 37 °C and 7.3% CO₂ prior analysis.

2.2. Morphology and immunohistochemistry

LEM and NEM sections were washed in phosphate-buffered saline (PBS), fixed with 4% formaldehyde and paraffin-embedded. Sections were cut at 5 µm thickness, deparaffinized, rehydrated, and stained with haematoxylin and eosin (HE). Immunohistochemical analysis was performed for Keratins 10, 16 and Ki67. Heat-mediated antigen retrieval was performed in citrate buffer pH-6 followed by blocking non-specific binding using PBS containing 1% bovine serum albumin (BSA, Sigma) and 2% normal human serum (NHS, Sanquin, Leiden, the Netherlands). Primary antibodies were diluted in PBS + 1%BSA + 2%NHS and incubated overnight at 4 °C. After washing with PBS sections were incubated with streptavidin-biotin-peroxidase complex system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. After washing in PBS, the signal was developed with 3,3'-diaminobenzidine (DAB) solution, and all slides were counterstained with haematoxylin. As negative controls, tissue sections were processed under the same experimental conditions as described above, except that they were incubated overnight at 4 °C in PBS + 1%BSA + 2%NHS without primary antibody.

2.3. Immunofluorescence

Cells were grown on glass coverslips and exposed to chemicals at CV 80% concentrations. After 24 h cells were fixed with 4% formaldehyde for 15 min at room temperature (all of the following steps were done at room temperature) and permeabilized in 0.2% Triton X-100-phosphate-buffered saline for 5 min. Cells were washed with phosphate-buffered saline and blocked with 5% goat serum for 1 h, incubated with primary antibody (anti-Nrf2 rabbit polyclonal, 1:500, Santa Cruz, CA, USA) for 1 h, washed, and incubated with secondary antibodies for 30 min, following extensive washing. All sections were counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Invitrogen) for 2 min. Sections were mounted with Vectashield

(Brunschwig chemie, Burlingame, USA), covered and visualized with a Leica DM5000 B microscope.

2.4. WST proliferation assay

Cell viability of monolayers was measured using the WST-1 assay (Roche diagnostics, Mannheim, Germany). Briefly, cells were seeded in 96-well flat-bottom plates (Greiner Bio-One, Frickenhausen, Germany) in 100 µl of culture medium 24–48 h prior to chemical treatment. After 24 h of chemical treatment, 10 µl of WST-1 reagent was added and plates were further incubated up to 3 h at 37 °C. Absorbance was measured at 450 nm using a plate reader (TECAN infinite F200, Tecan, Austria) in order to calculate the cell viability at 80%.

2.5. MTT proliferation assay

Twelve days air-exposed LEMs or NEMs were covered with an 11 mm filter paper disc (Finn Chamber, Phoenix, USA). Different concentrations of chemicals were applied onto the filters and incubated for 24 h and thereafter washed away with PBS. LEMs or NEMs were then incubated with thiazolyl blue tetrazolium bromide (MTT), (Sigma). MTT stock of 5 mg/ml PBS (filter sterilized) was diluted to a final concentration of 1 mg/ml in colorless DMEM medium. MTT medium was added to the models and incubated for 3 h at 37 °C. Viable cells will be able to reduce the tetrazolium dye to its insoluble formazan that gives a purple color. After 3 h the MTT is removed and washed away with PBS. After drying, the formazan was dissolved in 2 ml of isopropanol overnight at room temperature. Absorbance was measured at 570 nm using a plate reader (TECAN infinite F200) in order to calculate the cell viability at 80%.

2.6. Nuclear fractionation assay

Nuclear fractions of monolayer cultures and reconstructed epidermis were isolate using a Nuclear extract kit (Active motif, Carlsbad, CA) according to the manufacturer's protocol. Nuclear fractions were subjected to Western blot analysis.

2.7. Western blot analysis

Monolayer cells were lysed in M-Per buffer (Thermo Scientific, Rockford, USA), reconstructed epidermis was cut in pieces and lysed in T-Per lysis buffer (Thermo Scientific, Rockford, USA) both supplemented with protease inhibitor cocktail EDTA-free and HALT™ phosphatase inhibitors (Thermo Scientific). Cell lysates were measured using the BCA™ protein assay kit (Thermo Scientific). Equal amounts of proteins were separated by SDS–page and were transferred onto PVDF- transfer membranes (Thermo Scientific). Membranes were blocked in PBS, 0.1% Tween-20 (PBST) containing non-fat dry milk (ELK, Campina, The Netherlands) and subsequently incubated with the appropriate primary antibody. Membranes were washed four times with PBST and incubated with either horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:2500, Pierce Thermo Scientific, Rockford, USA). After four times washing with PBST, proteins were detected by enhanced chemi-luminescence (Super signal West femto (Pierce Thermo Scientific, Rockford, USA) and visualized by exposure to X-ray film (Fujifilm Europe GmbH, Düsseldorf, Germany).

2.8. Analysis of gene expression by quantitative PCR

Total RNA was prepared using RNeasy mini kit (Qiagen) following the manufacturer's protocol. RNA was quantified using a Nanodrop ND-1000 spectrophotometer. First- strand cDNA

synthesis was derived from 1 µg of total RNA using the iscript cDNA synthesis kit (Biorad) according to the manufacturer's protocol. Primers were tested in normal KC cDNA. Stability of reference genes was analyzed with the GeNorm method (Vandesompele et al., 2002). Expression analysis was performed with the BioRad software (iQ5) and was based on the delta delta Ct method with the reference genes that were most stably expressed according to the GeNorm analysis.

Expression levels of the following genes were measured: HMOX-1 (Hemeoxygenase-1), GCLM (Glutamate-cysteine ligase modifier subunit), TXNRD1 (Thioredoxin reductase 1), OSGIN-1 (oxidative stress induced growth inhibitor 1), B2 M (Beta-2 microglobulin), GUSB (Glucuronidase beta) and TBP (TATA binding protein). Quantitative real-time PCR was determined by using the following primer sets: following primer sets:

5'-TTCTCTGGCTGGCTTCCTTACC and
3'-CCATAGGCTCCTTCCTCTTTCC (*HMOX-1*),
5'-TATAAAGACAAACCAAGTAAATCTGAAAGGAG-3' and
3'-TGGATGGAGGTGACAGTAGTTGC-5' (*GCLM*),
5'-ACCTGCGTGCCTGTGCTTAC-3' and
3'-TGCCTTCTATTCACCAACTCTATCC-5' (*TXNRD1*),
5'-TGCTGCAGAGGAAGCTCAC-3' and
3'-TCGGACAGGTAGTCCAGGTC-5' (*OSGIN1*),
5'-GATGAGTATGCCTGCCGTGTG-3' and
3'-CAATCCAATGCGGCATCT-5' (*B2M*),
5'-CTCATTGGAATTTGCCGATT-3' and
3'-CCGAGTGAAGATCCCCTTTTA-5' (*GUSB*),
5'-CACGAACCACGGCACTGATT-3' and
3'-TTTCTTGCTGCCAGTCTGGAC-5' (*TBP*).

Real-Time PCRs were performed in 10 µl reaction volumes using the MyIQ system and SYBR Green PCR Master-mix (Biorad) according to the manufacturer's protocol.

The PCR cycles were: 3 min at 95 °C to activate the polymerase, 40 cycles of 20 s at 95 °C and 40 s at 60 °C, followed by the generation of a melt curve.

All qPCR values were averaged relative to the three different reference genes: B2 M, GUSB, TBP. For each data point, PCRs were performed in duplicate and values represent means ± standard deviation. For each gene the expression due to vehicle exposure was set at 1 and the expression induced by chemicals were normalized versus the vehicle.

2.9. Statistical analyses

Data were analyzed for statistical significance using the student t-test. *P* values of ≤ 0.05 were considered significant.

2.10. Antibodies, drugs and chemicals

The following antibodies were used: anti-Nrf2 rabbit polyclonal, 1:500 and 1:100 (Santa Cruz, CA, USA), anti-Tubulin rabbit polyclonal, 1:2000 (Abcam, Cambridge, UK), anti-B-actin rabbit polyclonal, 1:2000 (Cell Signaling, MA, USA). Secondary antibodies: Rhodamine Red™-X-conjugated goat anti-Rabbit 1:300 (Jackson ImmunoResearch Europe, Suffolk, UK). Drugs: MG132 (Calbiochem) was used as a proteasome inhibitor for 6 h prior to lysis at a concentration of 3 µM. Chemicals: 2,4-dinitrochlorobenzene (DNCB) and Sodium Dodecyl Sulfate (SDS) were purchased from Sigma. Monolayer KCs were exposed to DNCB at 10 µM while for epidermal models 1.25 mM was used. SDS was used at a concentration of 80 µM and 10 mM respectively for monolayers and epidermal models. All concentrations used resulted in 80% cell viability (determined with WST and MTT assay).

3. Results

3.1. NEMs show similar epidermal morphogenesis as LEMs

To evaluate whether NEMs show comparable characteristics as the LEMs, we examined the overall morphology and used immunohistochemical detection to determine the expression of the early differentiation marker Keratin 10 (K10), hyper-proliferation associated marker K16, and epidermal proliferation by Ki-67. As shown in Fig. 1A, the epidermis generated with N/TERT cell line contains all different layers (the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum) as found in LEMs and native human skin (data not shown). The expression of K10 is present in all suprabasal cell layers and absent in the basal layers, indicating the presence of a normal early differentiation program (Fig. 1B). The epidermis also shows a very weak expression of the hyper proliferation-associated marker K16 in the upper layers of the epidermis (Fig. 1C), indicating a normalized epidermis. NEMs contain proliferating basal KCs (Fig. 1D) (15 ± 5 Ki67 positive cells/100 basal KCs), which is comparable to LEMs and native skin (data not shown).

3.2. Nrf2 translocation occurs in primary KCs and in the N/TERT cell line

Having demonstrated the similarity in the morphological characteristics between NEMs and LEMs, we investigated how both cell types in monolayer cultures and reconstructed epidermis respond to chemical sensitizers. For this purpose cell cultures of both N/TERT and primary KCs were treated without ($t = 0$) or with 2,4-dinitrochlorobenzene ((DNCB, 10 μ M), a strong skin sensitizer) and Sodium dodecyl sulfate ((SDS, 80 μ M), a skin irritant) as a negative control for 1 or 2 h. We found that after treatment of monolayer KCs with DNCB a substantial amount of Rhodamine labeled Nrf2 protein was translocated to the cell nucleus, which was more than cells treated with SDS (Fig. 2A). Furthermore immunohistochemical analyses of NEMs treated with DNCB (Fig. 2b) also showed higher levels of Nrf2 in the nuclei compared to SDS treated epidermal models (Fig. 2c). Similar results were obtained with the LEMs (data not shown). Subsequently we evaluated whether Nrf2 expression could be stabilized by inhibiting the proteasome that is responsible for a constitutively degradation of Nrf2 using protea-

some inhibitor MG132 as a positive control for Nrf2 translocation. In addition we used a second approach to demonstrate Nrf2 translocation to the nucleus. For this purpose, we prepared nuclear extracts of monolayer cells cultures and epidermal models and subjected them to Western blot analysis in order to detect translocated Nrf2 protein levels in the nuclei (Fig. 3). As shown in Fig. 3a, we clearly observe elevated levels of Nrf2 in monolayer primary KCs treated with DNCB and MG132 as well as in N/TERT monolayer cultures (Fig. 3b). We could also confirm this in the LEMs (Fig. 3c) and NEMs (Fig. 3d).

3.3. Elevated expression of total protein levels of Nrf2 in primary KCs and the N/TERT cell line

We determined the total protein levels of Nrf2 in monolayer cultures of primary and N/TERT KCs as well as the NEMs and LEMs. After having shown that Nrf2 is prevented from degradation after DNCB treatment, we expected that Nrf2 protein levels stabilize after treatment. To that extend Western blot analysis was performed on all four KC-based models in order to detect endogenous levels of Nrf2. As depicted in Fig. 4 we observed an elevated protein expression of Nrf2 after DNCB treatment compared to SDS treated cells in a time dependent manner. Again vehicle control at time point 120 min is shown since earlier time points resulted in similar expression levels (data not shown). When comparing monolayer primary KCs (Fig. 4a) and monolayer N/TERT (Fig. 4b) we demonstrate that in both cell types the Nrf2 levels are induced when treated with DNCB. LEMs (Fig. 4c) and NEMs (Fig. 4d) also showed increased levels of Nrf2 after DNCB treatment. These results indicate that the sensitizer DNCB at least prevent Nrf2 degradation and enhances its expression levels.

3.4. Elevated mRNA expression levels of Nrf2 dependent genes after sensitization

Next, we explored the transcriptional activity of Nrf2. It is well known that genes that have an ARE consensus in their promoter region can be activated by transcription factor Nrf2. We therefore selected a subset of genes (HMOX-1, OSGIN-1, TXRND-1 and GCLM) that are described in literature to be transcriptional activated by Nrf2 in order to evaluate their mRNA levels after

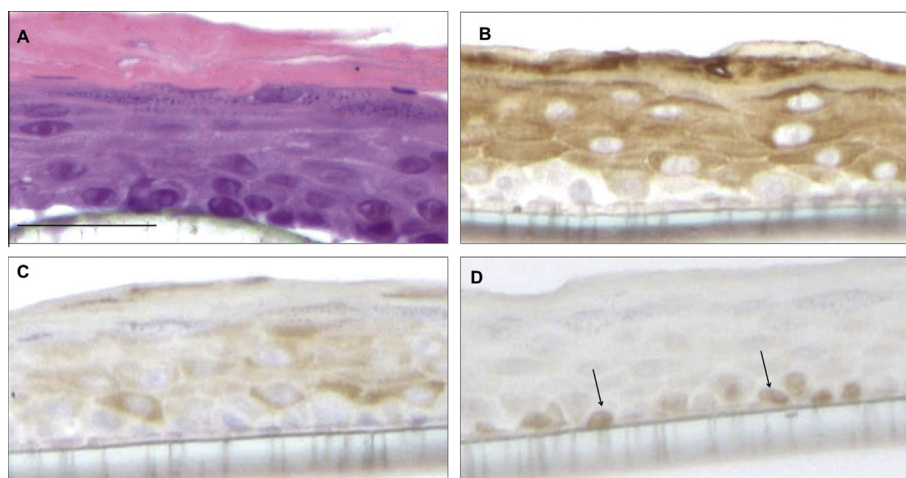


Fig. 1. Morphology and differentiation of NEMs. (A) Shown are cross sections of a Haematoxylin and Eosin (HE) staining on NEMs that have been cultured for 12 days at the air liquid interface. (B) Immunohistochemical staining showing the presence of K10 in the suprabasal layers and a very weak expression of the hyper-proliferation associated marker K16 (C) in the upper layers. Cells positive for the proliferation marker Ki67 are detected in the basal layer (arrows) (D). Scale bar represents 25 μ m.

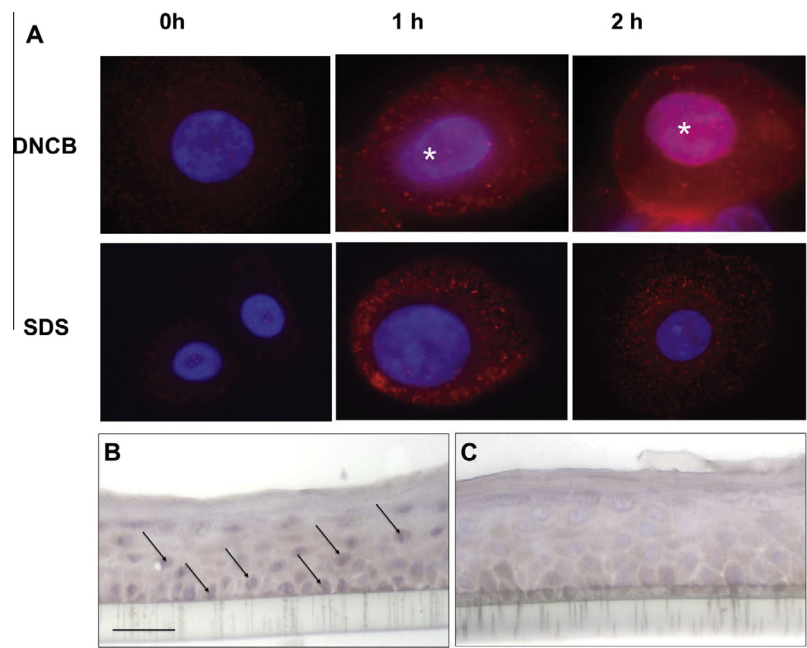


Fig. 2. Nrf2 nuclear translocation after DNCB exposure of KCs. (A) Monoculture of primary KCs were treated with either DNCB (sensitizer) or SDS (irritant) for 0, 1 or 2 h. Immunofluorescence staining was performed, cells were labeled with primary Nrf2 and visualized with secondary Rhodamine Red (red) antibody. Nuclei were visualized using DAPI staining (blue). Nuclear translocation of Nrf2 was clearly visible after treatment with DNCB (see asterisk). NEMs cultured for 12 days air-exposed were treated with DNCB, (B) and with SDS (C) for 4 h, following Immunohistochemical analysis of Nrf2 on paraffin embedded sections. Nrf2 was mainly translocated to the nucleus after DNCB treatment (arrows). Scale bar represents 50 μ m.

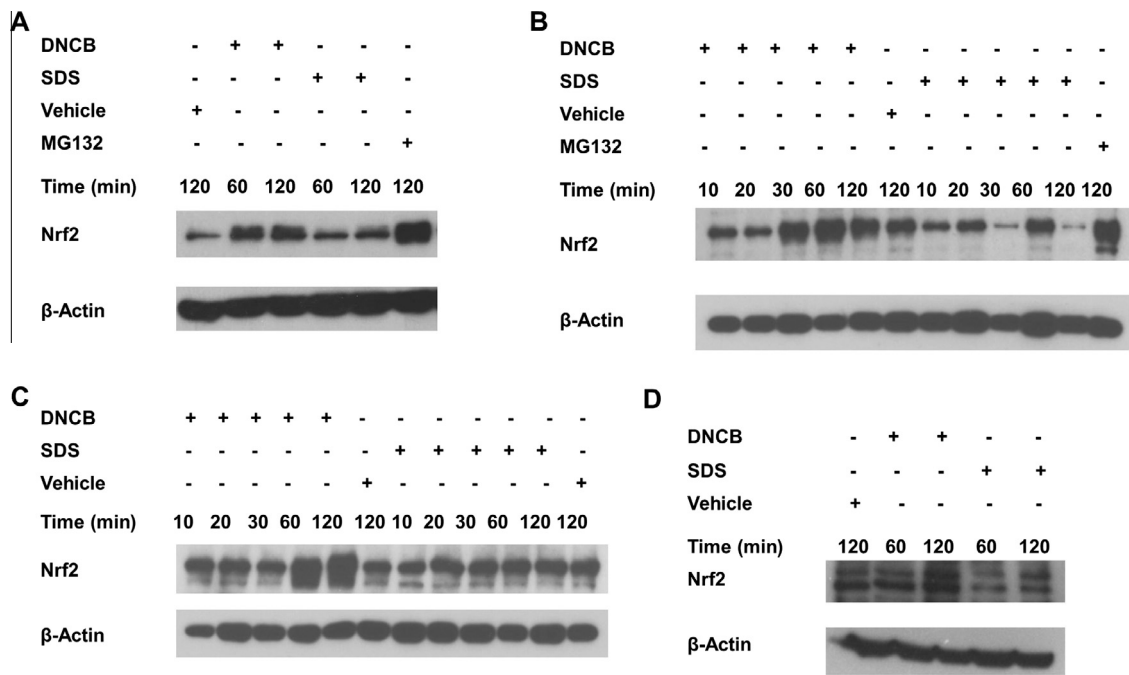


Fig. 3. Nuclear fractionation of KCs after treatment with DNCB and SDS. Monolayer of primary KCs (A) and monolayer of the N/TERT cell line (B) were treated at different time points with either DNCB or SDS. Vehicle control is shown at time point 120 min, earlier time points resulted in similar expression levels (data not shown). Proteasome inhibitor MG132 was used as a positive control. LEMs (C) and NEMs (D) were treated in the same way. Nuclear lysates were isolated and subjected to Western blot analysis in order to detect Nrf2 protein levels. Enhanced levels of Nrf2 were detected in all DNCB treated KCs and epidermal models. In all experiments b-actin was used as a loading control. Results represent three independent experiments.

treatment with either DNCB or SDS. First we compared the activation of these genes in monocultures of primary and N/TERT KCs (Fig. 5a and b). Expression of HMOX-1, OSGIN-1 and GCLM was

significantly upregulated (4 to 6-fold) in primary KCs (Fig. 5a), while in N/TERT monolayer cultures HMOX-1, OSGIN-1, TXNRD-1 and GCLM were significantly upregulated (6 to 9-fold) (Fig. 5b).

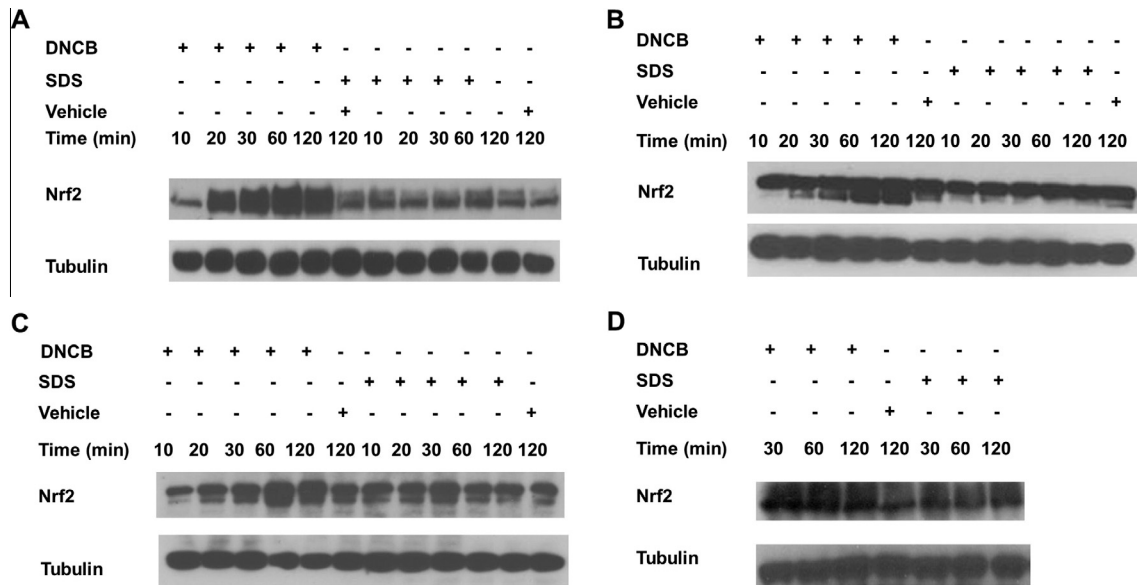


Fig. 4. Nrf2 protein expression levels in primary KCs and the N/TERT cell line. Monolayer of primary KCs (A) and monolayer of the N/TERT cell line (B) were treated at different time points with either DNCB or SDS. Vehicle control is shown at time point 120 min since earlier time points resulted in similar expression levels (data not shown). LEMs (C) and NEMs (D) were treated with either DNCB or SDS in a time dependent manner. Total lysates were prepared after chemical exposure and subjected to Western blot analysis in order to detect Nrf2 protein levels. Enhanced levels of Nrf2 were detected in all DNCB treated KCs and epidermal models. In all experiments tubulin was used as a loading control. Results represent three independent experiments.

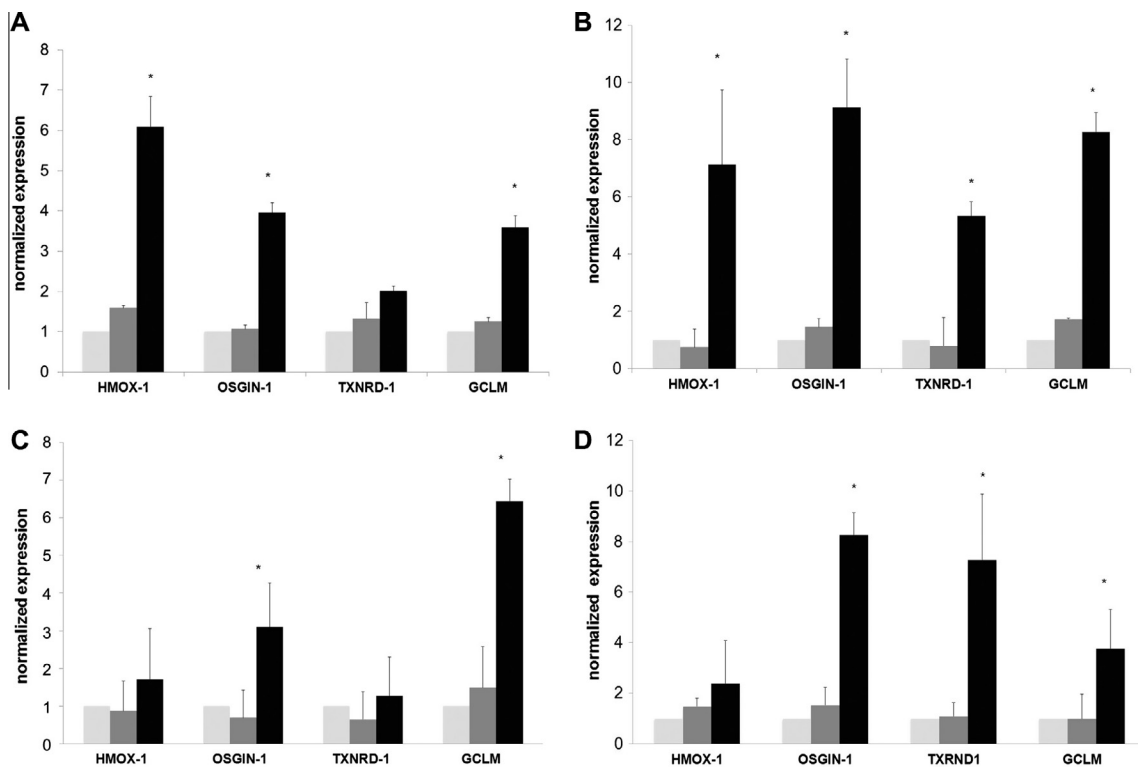


Fig. 5. Elevated mRNA expression of Nrf2 depended genes after treatment with DNCB or SDS. Monolayer of primary KCs (A); monolayer of the N/TERT cell line (B); NEMs (C); and ex vivo epidermal sheets (D). All different KC models were treated with DNCB or SDS for 4 h either in aqueous solution in monolayers or topical in epidermal models. RNA was isolated and q-PCR analysis was performed. Gene expression was normalized against reference genes GUSB, B2 M and TBP. Filled bars represent DNCB, grey bars represent SDS and white bars represent vehicle. Error bar show SEM. * $P < 0.05$. Results represent three independent experiments.

In addition the same set of genes was also tested under the same conditions in NEMs and ex vivo human skin (Fig. 5c and d). GCLM (approx. 5-fold) and OSGIN-1 (approx. 3.5-fold) were significantly upregulated in NEMs in response to DNCB (Fig. 5c). However, when analyzing the genes in ex vivo skin we could measure elevated

mRNA expression of OSGIN-1, TXNRD-1 and GCLM, while HMOX-1 expression was not upregulated (Fig. 5d). This result indicates that most of the tested Nrf2 depended genes are significantly activated in the monolayer and epidermal models after sensitization but not in response to the irritant SDS.

4. Discussion

Commercial available human reconstructed epidermal models have been previously used to discriminate sensitizers from irritants (McKim et al., 2012; Gibbs et al., 2013a,b; Saito et al., 2013a,b). It is known that donor-to-donor variability of primary cell culture models result in inconsistencies that makes the interpretation of the data difficult. In order to minimize this donor variability, we believe that the use of an epidermal model generated with a cell line would be of added value for screening purposes. Moreover, fresh human skin needed for construction of primary human skin equivalents is often difficult to obtain and the use of a cell line can overcome this problem.

In this study we present the use of the N/TERT keratinocyte cell line as monolayer and epidermal model (NEM) as a possible tool to discriminate sensitizers from irritants. In addition, we compared the responses of N/TERT and primary human keratinocytes to a sensitizer and an irritant.

First, we tested the N/TERT cell line for its ability to generate an epidermal model consisting of a proper barrier with properties comparable to our LEMs (El Ghalbzouri et al., 2008; Boelsma et al., 2000; Ponc et al., 2000a,b; Ponc et al., 2002). Morphological and immunohistochemical analyses demonstrated high similarity between LEMs and NEMs. To our knowledge this N/TERT keratinocyte cell line is (apart from the NIKS cell line only available as a commercial epidermal model, Rasmussen et al., 2010; Centanni et al., 2011), the only epidermal model, which can generate an epidermis with a competent barrier comparable to our in house LEMs (*manuscript in preparation*).

Biological key events within skin sensitization are defined as Adverse Outcome Pathway (AOP). These are molecular activation steps that mimic the whole skin sensitization process, presented by the OECD (Organization for Economic Co-operation and Development) as a strategy to develop integrated *in vitro* assays. One of these key events is the response of keratinocytes (OECD, 2012a,b).

Sensitizers can penetrate into the skin and covalently bind to endogenous proteins in the skin, thereby inducing the production of reactive oxygen species (ROS) by a yet unknown mechanism. Based on this first key event, tests are in development, such as the THP-1 cell line (based on ROS production) showing the ability to distinguish sensitizers from irritants (Esser et al., 2012; Saito et al., 2013a,b). The second key event is the activation of pathways and cytokine production in keratinocytes in response to sensitizers. Covalent binding of skin proteins eventually leads to activation of the Keap1-Nrf2-ARE pathway in keratinocytes, which in turn leads to the expression of several target genes. At present several *in vitro* models have been developed based on this key event such as the Keratinosens®. The Keratinosens® assay is a HaCat keratinocyte cell line with an incorporated luciferase reporter and based on transcriptional activation of Nrf2 targets (Emter et al., 2010). Our presented 3D-NEM would fit as an integrated assay in the second key event in skin sensitization and could provide an important added value.

Based on literature we selected four Nrf2 targets HMOX-1, TXRND-1, OSGIN-1 and GCLM (Ade et al., 2009; Natsch and Emter, 2008; Vandebriel and van Loveren, 2010; van der Veen et al., 2013). We found HMOX-1 to be differentially upregulated in monolayer primary KCs and N/TERTs which is in accordance with previous studies using several different monolayer cell lines, such as THP-1 cells, Dendritic cells and HaCat KCs (Ade et al., 2009; Vandebriel and van Loveren, 2010; van der Veen et al., 2013). However, we could not observe any significant upregulation of the HMOX-1 gene in NEMs, nor in *ex vivo* sensitized skin after exposure with sensitizer DNCB. Interestingly, staining for HMOX-1 protein levels in both LEMs and NEMs showed constitutive expression of HMOX-1 in the granular layer (data not shown), which suggests an altered expression pattern for HMOX-1 in

stratified epidermis (i.e. expressed only by differentiated KCs). TXRND-1 was significantly upregulated in N/TERT monolayers and *ex vivo* skin but not significantly different in primary KCs and LEMs. OSGIN-1 and GCLM were significantly upregulated after sensitization irrespective of the model used. We therefore propose that OSGIN-1 and GCLM might serve as potential biomarkers for identification of sensitizers in NEMs.

The LLNA is currently the preferred test for the identification of sensitizers. However, in this test SDS, being an irritant for humans, is identified as a false positive (Kimber and Dearman, 2002; Basketter and Kimber, 2011). We observed that SDS is not activating the Keap1-Nrf2-ARE pathway in our models and cell lines which is in line with the results obtained using the Keap1-Nrf2-ARE pathway in the Keratinosens® assay (Emter et al., 2010) and correlating well with human clinical data. In addition the Dendritic Cell migration assay was also able to classify SDS correctly as a non-sensitizer (Gibbs et al., 2013a). The inaccuracy of the LLNA might be due to species differences (human versus mice) or the false positivity of SDS might be occurring at a later stage of the process of initiation of ACD (T-cell proliferation).

Although the NEMs seem to be a very promising tool for chemical identification, a larger battery of chemicals should be tested in order to prove the reproducibility of the candidate genes as possible biomarkers in NEMs for skin sensitization. To that extend it would be of great interest to further explore the responses of the NEMs to sensitizers and non-sensitizers and identify possible additional biomarkers. The N/TERT cell line can be used as monolayer for aqueous soluble chemicals and as an epidermal model for non-aqueous soluble chemicals for topical application, or chemicals that need to be metabolically converted.

Already there has been an intra-laboratory validation of four *in vitro* models addressing three different key events during the induction of skin sensitization. These are the (1) Direct Peptide Reactivity Assay (DPRA) (Gerberick et al., 2004), (2) ARE dependent gene activity in the HaCat cell line (Keratinosens/LuSens), (Emter et al., 2010) and (3) Dendritic cell activation assays based on U-937 and THP-1 (Ahikaga et al., 2006; Python et al., 2007; Ade et al., 2009; Saito et al., 2013a,b). These test have been reviewed in the paper Bauch et al., 2012 resulting in a prediction accuracy range between 71% and 99%. The fourth key event (T-cell proliferation) was not included in this validation. With regards to the AOP, our model could fit well as an integrated assay in the second key events of skin sensitization.

Although the Keap1-Nrf2-ARE pathway activation is included in this validation, we believe our model has an added value as it offers a 3D microenvironment and thereby mimicking the *in vivo* situation. In addition, this second key event can be extended by investigating biomarkers on cytokine levels. Moreover, when testing more chemicals it could be necessary to combine the N/TERT other cell type models in order to increase the prediction range.

Overall, our work demonstrates that the N/TERT is a promising reproducible *in vitro* tool that can be further developed for skin sensitizer identification. Most importantly is that the N/TERT cell line enables the reconstruction of an epidermis comparable to our in house LEMs, which overcomes the need of primary human skin tissue. We therefore aim to further validate the NEMs using a larger set of chemicals.

Conflict of Interest

None declared.

Transparency Document

The [Transparency document](#) associated with this article can be found in the online version.

Acknowledgments

This research was supported by a grant of the Netherlands Genomics.

Initiative/Netherlands Organization for Scientific Research (NWO) No.: 050-060-510.

HMOX-1 primers were kindly provided by Dr. Pieter van der Velden (department of Ophthalmology, LUMC). We would like to thank David Janson for his technical assistance during this study.

References

- Ade, N., Leon, F., Pallardy, M., Peiffer, J.L., Kerdine-Romer, S., Tissier, M.H., Bonnet, P.A., Fabre, I., Ourlin, J.C., 2009. HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: role of the Keap1/Nrf2 pathway. *Toxicol. Sci.* 107, 451–460.
- Ahikaga, T., Yoshida, Y., Hirota, M., Yoneyama, K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H., Toyoda, H., 2006. Development of an in vitro skin sensitization test using human cell lines: the human cell line activation test (h-CLAT) I. Optimization of the h-CLAT protocol. *Toxicol. Vitro* 20 (5), 767–773.
- Basketter, D.A., Kimber, I., 2011. Skin irritation, false positives and the local lymph node assay: a guideline issue? *Regul. Toxicol. Pharmacol.* 61, 137–140.
- Basketter, D.A., Gerberick, F., Kimber, I., 2007. The local lymph node assay and the assessment of relative potency: status of validation. *Contact Dermatitis*, 57, 70–75.
- Bauch, C., Kolle, S.N., Ramirez, T., Eltze, T., Fabian, E., Mehling, A., Teubner, W., van Ravenzwaay, B., Landsiedel, R., 2012. Putting the parts together: combining in vitro methods to test for skin sensitizing potentials. *Regul. Toxicol. Pharmacol.* 63, 489–504.
- Boelsma, E., Gibbs, S., Faller, C., Ponc, M., 2000. Characterization and comparison of reconstructed skin models: morphological and immunohistochemical evaluation. *Acta Derm. Venereol.* 80, 82–88.
- Cavani, A., 2008. Immune regulatory mechanisms in allergic contact dermatitis and contact sensitization. *Chem. Immunol. Allergy* 94, 93–100.
- Centanni, J.M., Straseski, J.A., Wicks, A., Hank, J.A., Rasmussen, C.A., Lokuta, M.A., Schurr, M.J., Foster, K.N., Faucher, L.D., Caruso, D.M., Comer, A.R., Allen-Hoffmann, B.L., 2011. StrataGraft skin substitute is well-tolerated and is not acutely immunogenic in patients with traumatic wounds: results from a prospective, randomized, controlled dose escalation trial. *Ann. Surg.* 253, 672–683.
- Corsini, E., Mitjans, M., Galbiati, V., Lucchi, L., Galli, C.L., Marinovich, M., 2009. Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicol. In Vitro* 23, 789–796.
- Dickson, M.A., Hahn, W.C., Ino, Y., Ronfard, V., Wu, J.Y., Weinberg, R.A., Louis, D.N., Li, F.P., Rheinwald, J.G., 2000. Human keratinocytes that express hTERT and also bypass a p16^{INK4a}-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol. Cell. Biol.* 20, 1436–1447.
- El Ghalbzouri, A., Siamari, R., Willemze, R., Ponc, M., 2008. Leiden reconstructed human epidermal model as a tool for the evaluation of the skin corrosion and irritation potential according to the ECVAM guidelines. *Toxicol. In Vitro* 22, 1311–1320.
- Emter, R., Ellis, G., Natsch, A., 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. *Toxicol. Appl. Pharmacol.* 245, 281–290.
- Esser, P.R., Wölfe, U., Dürr, C., von Loewenich, F.D., Schempp, C.M., Freudenberg, M.A., Jakob, T., Martin, S.F., 2012. Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PLoS ONE* 7, e41340.
- Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W., Lepoittevin, J.P., 2004. Development of a peptide reactivity assay for screening contact allergens. *Toxicol. Sci.* 81, 332–343.
- Gibbs, S., Spiekstra, S.W., Corsini, E., McLeod, J., Reinders, J., 2013a. Dendritic cell migration assay: a potential prediction model for identification of contact allergens. *Toxicol. In Vitro* 27, 1170–1179.
- Gibbs, S., Corsini, E., Spiekstra, S.W., Galbiati, V., Fuchs, H.W., DeGeorge, G., Troese, M., Hayden, P., Deng, P., Roggen, E., 2013b. An epidermal equivalent assay for identification and ranking potency of contact sensitizers. *Toxicol. Appl. Pharmacol.* 272, 529–541.
- Gober, M.D., Gaspari, A.A., 2008. Allergic contact dermatitis. *Curr. Dir. Autoimmun.* 10, 1–26.
- Gotz, C., Pfeiffer, R., Tigges, J., Blatz, V., Jackh, C., Freytag, E.M., Fabian, E., Landsiedel, R., Merk, H.F., Krutmann, J., Edwards, R.J., Pease, C., Goebel, C., Hewitt, N., Fritsche, E., 2012. Xenobiotic metabolism capacities of human skin in comparison with a 3D epidermis model and keratinocyte-based cell culture as in vitro alternatives for chemical testing: activating enzymes (Phase I). *Exp. Dermatol.* 21, 358–363.
- Hewitt, N.J., Edwards, R., Fritsche, E., Goebel, C., Aeby, P., Scheel, J., Reisinger, K., Ouedraogo, G., Duche, D., Eilstein, J., Latil, A., Kenny, J., Moore, C., Kuehn, J., Barroso, J., Fautz, R., Pfuhrer, S., 2013. Use of human in vitro skin models for accurate and ethical risk assessment: metabolic considerations. *Toxicol. Sci.* [Epub ahead of print].
- Hu, T., Khambatta, Z.S., Hayden, P.J., Bolmarcich, J., Binder, R.L., Robinson, M.K., Carr, G.J., Tiesman, J.P., Jarrold, B.B., Osborne, R., Reichling, T.D., Nemeth, S.T., Aardema, M.J., 2010. Xenobiotic metabolism gene expression in the EpiDerm in vitro 3D human epidermis model compared to human skin. *Toxicol. In Vitro* 24, 1450–1463.
- Kaplan, D.H., Igyártó, B.Z., Gaspari, A.A., 2012. Early immune events in the induction of allergic contact dermatitis. *Nat. Rev. Immunol.* 12, 114–124.
- Kimber, I., Dearman, R.J., 2002. Allergic contact dermatitis: the cellular effectors. *Contact Dermatitis* 46, 1–5.
- Kimber, I., Dearman, R.J., Scholes, E.W., Basketter, D.A., 1994. The local lymph node assay: developments and applications. *Toxicology* 93, 13–31.
- Kollisch, G., Kalali, B.N., Voelcker, V., Wallich, R., Behrendt, H., Ring, J., Bauer, S., Jakob, T., Mempel, M., Ollert, M., 2005. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology* 114, 531–541.
- McKim, J.M., Keller, D.J., Gorski, J.R., 2010. A new in vitro method for identifying chemical sensitizers combining peptide binding with ARE/EpRE-mediated gene expression in human skin cells. *Cutan. Ocul. Toxicol.* 29, 171–192.
- McKim, J.M., Keller, D.J., Gorski, J.R., 2012. An in vitro method for detecting chemical sensitization using human reconstructed skin models and its applicability to cosmetic, pharmaceutical, and medical device safety testing. *Cutan. Ocul. Toxicol.* 31, 292–305.
- Natsch, A., 2010. The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers functional relevance and a hypothesis on innate reactions to skin sensitizers. *Toxicol. Sci.* 113, 284–292.
- Natsch, A., Emter, R., 2008. Skin sensitizers induce antioxidant response element dependent genes: application to the in vitro testing of the sensitization potential of chemicals. *Toxicol. Sci.* 102, 110–119.
- Neis, M.M., Wendel, A., Wiederholt, T., Marquardt, Y., Joussen, S., Baron, J.M., Merk, H.F., 2010. Expression and induction of cytochrome p450 isoenzymes in human skin equivalents. *Skin Pharmacol. Physiol.* 23, 29–39.
- Nestle, F.O., Di Meglio, P., Qin, J.Z., Nickoloff, B.J., 2009. Skin immune sentinels in health and disease. *Nat. Rev. Immunol.* 9, 679–691.
- Nickoloff, B.J., 2006. Keratinocytes regain momentum as instigators of cutaneous inflammation. *Trends Mol. Med.* 12, 102–106.
- OECD, 2012a. The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168. <[http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO\(2012\)10/PART1&docLanguage=En](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2012)10/PART1&docLanguage=En)>.
- OECD, 2012b. The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 2: Use of the AOP to Develop Chemical Categories and Integrated Assessment and Testing Approaches. Series on Testing and Assessment No. 168. <[http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO\(2012\)10/PART2&docLanguage=En](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2012)10/PART2&docLanguage=En)>.
- Ponc, M., Weerheim, A., Kempenaar, J., Mulder, A., Gooris, G.S., Bouwstra, J., Mommaas, A.M., 1997. The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. *J. Invest. Dermatol.* 109, 348–355.
- Ponc, M., Boelsma, E., Weerheim, A., 2000a. Covalently bound lipids in reconstructed human epithelia. *Acta Derm. Venereol.* 80, 89–93.
- Ponc, M., Boelsma, E., Weerheim, A., Mulder, A., Bouwstra, J., Mommaas, M., 2000b. Lipid and ultrastructural characterization of reconstructed skin models. *Int. J. Pharm.* 203, 211–225.
- Ponc, M., Boelsma, E., Gibbs, S., Mommaas, M., 2002. Characterization of reconstructed skin models. *Skin Pharmacol. Appl. Skin Physiol.* 15 (Suppl. 1), 4–17.
- Python, F., Goebel, C., Aeby, P., 2007. Assessment of the U937 cell line for the detection of contact allergens. *Toxicol. Appl. Pharmacol.* 220, 113–124.
- Rasmussen, C., Gratz, K., Liebel, F., Southall, M., Garay, M., Bhattacharyya, S., Simon, N., Vander Zanden, M., Van Winkle, K., Pirnstill, J., Pirnstill, S., Comer, A., Allen-Hoffmann, B.L., 2010. The StrataTest® human skin model, a consistent in vitro alternative for toxicological testing. *Toxicol. In Vitro* 24, 2021–2029.
- Saito, K., Miyazawa, M., Nukada, Y., Sakaguchi, H., Nishiyama, N., 2013a. Development of an in vitro skin sensitization test based on ROS production in THP-1 cells. *Toxicol. In Vitro* 27, 857–863.
- Saito, K., Nukada, Y., Takenouchi, O., Miyazawa, M., Sakaguchi, H., Nishiyama, N., 2013b. Development of a new in vitro skin sensitization assay (Epidermal Sensitization Assay; EpiSensa) using reconstructed human epidermis. *Toxicol. In Vitro* 27 (8), 2213–2224.
- Thakoersing, V.S., Ponc, M., Bouwstra, J.A., 2010. Generation of human skin equivalents under submerged conditions-mimicking the in utero environment. *Tissue Eng. Part A* 2010 (16), 1433–1441.
- Thakoersing, V.S., Gooris, G.S., Mulder, A., Rietveld, M., El Ghalbzouri, A., Bouwstra, J.A., 2012. Unraveling barrier properties of three different in-house human skin equivalents. *Tissue Eng. Part C Methods* 2012 (18), 1–11.
- van der Veen, J.W., Pronk, T.E., van Loveren, H., Ezendam, J., 2013. Applicability of a keratinocyte gene signature to predict skin sensitizing potential. *Toxicol. In Vitro* 27, 314–322.
- Vandebriel, R.J., van Loveren, H., 2010. Non-animal sensitization testing: state-of-the-art. *Crit. Rev. Toxicol.* 40, 389–404.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, 3.
- Vocanson, M., Hennino, A., Rozières, A., Poyet, G., Nicolas, J.F., 2009. Effector and regulatory mechanisms in allergic contact dermatitis. *Allergy* 64, 1699–1714.