



Gene expression changes induced by skin sensitizers in the KeratinoSens™ cell line: Discriminating Nrf2-dependent and Nrf2-independent events



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ARTICLE INFO

Article history:

Received 10 March 2013

Accepted 11 September 2013

Available online 20 September 2013

Keywords:

Skin sensitization

Gene expression

Keratinocytes

Small interfering RNA

Nrf2

KeratinoSens™

ABSTRACT

The KeratinoSens™ assay is an *in vitro* screen for the skin sensitization potential of chemicals. It is based on a luciferase reporter gene under the control of the antioxidant response element of the aldo-ketoreductase gene *AKR1C2*. The transferability, reproducibility, and predictivity of the KeratinoSens™ assay have been investigated in detail and it is currently under assessment at the European Center for Validation of Alternatives to animal testing (ECVAM). Here we investigate the sensitizer-induced gene expression in the KeratinoSens™ cell line at the mRNA level and discriminate Nrf2-dependent and Nrf2-independent events by using siRNA to better characterize this test system at the molecular level. The results show that (i) the sensitizer-induced luciferase signal in KeratinoSens™ cells is completely dependent on Nrf2. The same holds true for the luciferase induction observed for the false positive chemical Tween80, indicating that the false positive result is not due to recruitment of an alternative transcription factor. (ii) Luciferase induction parallels the induction of endogenous Nrf2-dependent genes, indicating that the luciferase signal is representative for the sensitizer-induced Nrf2-response. (iii) The induction by sensitizers of additional genetic markers related to heat shock proteins and cellular stress could be reproduced in the KeratinoSens™ cell line and they were shown to be Nrf2-independent. These results confirm that the KeratinoSens™ cell line is a rapid and adequate screening tool to assess the sensitizer-induced Nrf2-response in keratinocytes.

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1. Introduction

The European ban of animal testing for cosmetics in 2013 has triggered large research initiatives with the aim to develop new testing strategies that predict skin sensitization, notably the Cosmetics Europe research program (Aeby et al., 2010), the European 6th framework program Sens-It-Iv (Rovida et al., 2007), and the program of the Netherlands Toxicogenomics Center (www.toxicogenomics.nl). One possibility to identify skin sensitizers is the examination of altered gene expression patterns or the selective

induction of signaling pathways in cell cultures challenged with test chemicals.

The aldo-ketoreductase gene *AKR1C2* gene was one of the first biomarkers that was found to be upregulated by sensitizers in dendritic cells (Gildea et al., 2006; Ryan et al., 2004) and later in keratinocytes (McKim et al., 2010). This gene contains a functional antioxidant response element (ARE) and is therefore dependent on Nrf2 for transcription (Lou et al., 2006). Based on this biomolecular evidence, we developed the KeratinoSens™ cell line which contains the ARE sequence from the human *AKR1C2* gene linked to a luciferase gene (Emter et al., 2010). The KeratinoSens™ assay was studied in detail for its predictivity, transferability and reproducibility (Natsch et al., 2011) and the assay is currently being assessed by ECVAM. As the assay moves forward in this assessment, a more detailed mechanistic understanding will be helpful to compare the reporter-gene approach to other methods, understand the response to false positives and false negatives and to find markers activated by other signaling pathways yielding potential additional information.

Abbreviations: LLNA, local lymph node assay; Nrf2, nuclear factor-erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; FCS, fetal calf serum; qRT-PCR, quantitative real time polymerase chain reaction; DMSO, dimethylsulfoxide; DNCB, 2,4-dinitrochlorobenzene; SDS, sodium dodecyl sulfate.

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A wide range of skin sensitizers induce the luciferase gene in this reporter cell line (Ball et al., 2011; Delaine et al., 2011; Emter et al., 2010; Natsch et al., 2013). Nonetheless, some false negative results have also been obtained (Natsch et al., 2013). Among these are several prohaptens, which require P450 mediated enzymatic oxidation to form the reactive species, and chemicals that transfer an acyl moiety especially to lysine-residues (different anhydrides such as phthalic anhydride, trimellitic anhydride and hexahydrophthalic anhydride or phenyl esters such as phenyl benzoate). In addition, some non-electrophilic false positives have been identified, including the non-ionic detergent Tween80. Also, sodium dodecyl sulfate (SDS) induces the luciferase yet only at low cellular viability.

The use of other genetic markers that have previously been identified in keratinocytes might add non-redundant information and thereby aid in correct classification, if they were induced by differentially regulated signaling pathways. A number of recently conducted studies have identified several promising genes and signaling pathways in dendritic cells and keratinocytes. In dendritic cell lines, p38 MAPK activation, induction of Nrf2/ARE-dependent genes, and changes in cell surface thiols are promising candidates for a platform of tests to predict the skin sensitisation hazard (Neves et al., 2011). In keratinocytes, two recent studies (Miyazawa et al., 2011; Vandebriel et al., 2010) showed that the Hsp70 response also has predictive potential. Four recent detailed gene-chip studies examined sensitizer-induced gene expression changes in dendritic cells (Johansson et al., 2011), keratinocyte cell lines (van der Veen et al., 2013; Vandebriel et al., 2010) and mouse skin (Miyazawa and Takashima, 2012). Using RT-PCR, gene expression was studied in HaCaT keratinocytes and epidermal models (McKim et al., 2010, 2012). One can conclude from these studies that the common significantly up-regulated pathway found is the Nrf2-pathway. Nevertheless, a broad range of other markers of interest was also identified.

So far, the involvement of Nrf2 in the luciferase induction has not been directly demonstrated in the recombinant KeratinoSens™ cell-line. In addition, the reporter gene response has yet to be directly compared to the induction of endogenous genes to evaluate whether the luciferase induction is a true reflection of the endogenous Nrf2-response to sensitizers. To further characterize the response of the KeratinoSens™ cell line, we first tested here whether the luciferase signal is indeed dependent on Nrf2. Next, the response was compared to the response of several endogenous Nrf2-dependent genes. Furthermore, we investigated whether emerging genetic markers in keratinocytes are also Nrf2-regulated in this cell line and whether they may add non-redundant information for correct classification, especially for the false negative chemicals.

2. Materials and methods

2.1. Test chemicals

The test chemicals were obtained from Sigma-Aldrich (Buchs Switzerland) and Givaudan Schweiz AG (Duebendorf, Switzerland). They included chemicals that are typically true positives and chemicals reported previous as either false positive or false negative in the KeratinoSens™ assay. Data on the chemicals and the rationale for their selection is summarized in Table 1. Test chemical concentrations, at which cells maintain >70% viability after 8 h, were selected for all the RT-PCR experiments.

2.2. Cell line and the standard KeratinoSens™ assay

The KeratinoSens™ cell line and method of the KeratinoSens™ assay have been previously described (Emter et al., 2010; Natsch et al., 2011). Unless otherwise indicated, the experiments were

performed according to the published procedure (cell density, incubation time, compound addition). Briefly, cells were seeded at a density of 10,000 cells per well in 96-well plates and grown for 24 h. The medium was then replaced with fresh medium containing 1% FCS, containing the test chemical and 1% DMSO. Cells were incubated for 48 h with the test agents, after which luciferase activity and cell viability, using the MTT assay (Mosmann, 1983), were determined.

2.3. RT-PCR experiments

Cells were seeded in 6-well plates at a density of 150,000 cells per well in Dulbecco's modified Eagle's medium containing Gluta-MAX™ (Gibco/Invitrogen) and 9% fetal calf serum (FCS). 24 h after seeding, the growth medium was replaced by fresh medium containing 1% FCS, the indicated concentrations of the test chemicals, and 1% DMSO. At the indicated times after compound addition, the cells were harvested by trypsinization. RNA was isolated using the RNeasy® Micro Kit (Qiagen, Hombrechtikon, Switzerland). RNA concentration was measured and 0.4 µg of each sample was used for cDNA synthesis using the QuantiTect® Reverse Transcription Kit (Qiagen). The levels of the specific cDNAs were determined using the Rotor-Gene Q, the Rotor-Gene™ SYBR® Green PCR Kit (Qiagen), and the primer pairs listed in the supplementary data (Table 1). Primers were obtained from Qiagen or synthesized by Microsynth (Balgach, Switzerland). Table 2 lists the rationale for the selection of and the literature reference for the genetic markers investigated in this study.

2.4. siRNA experiments

The transfection mix was prepared by mixing two mL of DMEM w/o FCS, 240 pmol All Stars negative control siRNA (Qiagen) or 240 pmol Nrf2-siRNA (Sense 5'-GUCCCAGUGUGGCAUCACCTT-3', Antisense 5'-GGUGAUGCCACACUGGGACTT-3') (Kimura et al., 2009), and 20 µL Lipofectamine RNAiMAX (Invitrogen). This mix was incubated at room temperature for 15 min. Then, 2×10^6 KeratinoSens™ cells were resuspended in 10 ml DMEM containing 9% FCS, added to the transfection mix and transferred to a 10 cm tissue culture dish. Three days later, the cells were harvested by trypsinization and further used for either the RT-PCR experiments as described above or for the luciferase-induction experiments according to the standard protocol (Emter et al., 2010).

3. Results

3.1. The luciferase signal induced by sensitizers in the KeratinoSens™ assay is dependent on Nrf2

In a first set of experiments, cells were pre-treated with Nrf2-siRNA or with control siRNA three days prior to seeding. The response of the siRNA treated cells to the extreme sensitizer 2,4-dinitrochlorobenzene (DNCB) and the two moderate sensitizers cinnamic aldehyde (CA) and (2E)-5,6,7-trimethyl-2,5-octadien-4-one was then measured in the KeratinoSens™ assay performed according to the standard method of the KeratinoSens™ assay. Upregulation of the luciferase signal by all three sensitizers was significantly reduced ($p < 0.005$) in the Nrf2-siRNA-treated cells as compared to the control siRNA-treated cells (Fig. 1). Treatment with Nrf2-siRNA reduced the sensitizer-induced luciferase signal by at least 80% at most test concentrations. The remaining signal can be attributed to the residual presence of Nrf2. At the time of compound addition (24 h after seeding), the knockdown of Nrf2 at the mRNA level was at $87 \pm 5\%$ in three independent experiments. At the end of the experiment (i.e. when the luciferase signal

Table 1
Test chemicals.

Name	Cas-Nr.	LLNA EC3	Historical KeratinoSens™ result and rationale for selection
2,4-dinitrochlorobenzene	97-00-7	0.04	Correct positive, extreme sensitizer
Cinnamic aldehyde	104-55-2	3.1	Correct positive, moderate sensitizer
(2E)-5,6,7-trimethyl-2,5-octadien-4-one	357650-26-1	1.6	Correct positive, moderate sensitizer
Sodium dodecyl sulfate	151-21-3	14, false positive	Correct negative according to the prediction model, luciferase induction in a small window of cytotoxic concentrations
Tween 80	9005-65-6	Non-sensitizer	False positive despite lack of electrophilic features
Phenyl benzoate	93-99-2	17.1	Acyl-transfer agent, weak sensitizer, false negative
Phthalic anhydride	85-44-9	0.16	Acyl-transfer agent specifically reactive with amine groups, respiratory sensitizer, and skin sensitizer according to animal tests, false negative vs. animal data

Table 2
Genetic markers investigated in the current study and rationale for their inclusion.

Gene	Coding for	Related pathways	Rationale ^a
NQO1	NAD(P)H dehydrogenase (quinone 1)	Oxidative stress and Nrf2	2
HMOX1	Heme oxygenase 1		2, 3, 5
SRXN1	Sulfiredoxin 1		5
ADM	Adrenomedullin		5
AKR1C2	Aldo-keto reductase family 1, member C2		1, 2
STC2	Stanniocalcin-2	Heat shock response	5
HSPA1A	Heat shock 70 kDa protein 1A		3, 4
HSPA1B	Heat shock 70 kDa protein 1B		3
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4		4
FOS	FBJ murine osteosarcoma viral oncogene homolog	General stress and immune response	5
FOSL1	Fos-like antigen 1		5
ATF3	Activating transcription factor 3		4
ANKRD37	Ankyrin repeat domain 37	Unknown	5
luc2	Recombinant luciferase in KeratinoSens™		

^aThe genetic markers were selected for the following reasons and from the following studies: 1, key sensitizer-upregulated gene in dendritic cells (Gildea et al., 2006); 2, Nrf2/Keap1-dependent gene in keratinocytes (MacLeod et al., 2009); 3, key up-regulated gene discriminating between sensitizers and non-sensitizers in HaCaT keratinocytes (Vandebriel et al., 2010); 4, key up-regulated gene discriminating between sensitizers and non-sensitizers in epidermis model (Miyazawa et al., 2011); 5, key up-regulated gene discriminating between sensitizers and non-sensitizers in HaCaT keratinocytes (van der Veen et al., 2013)

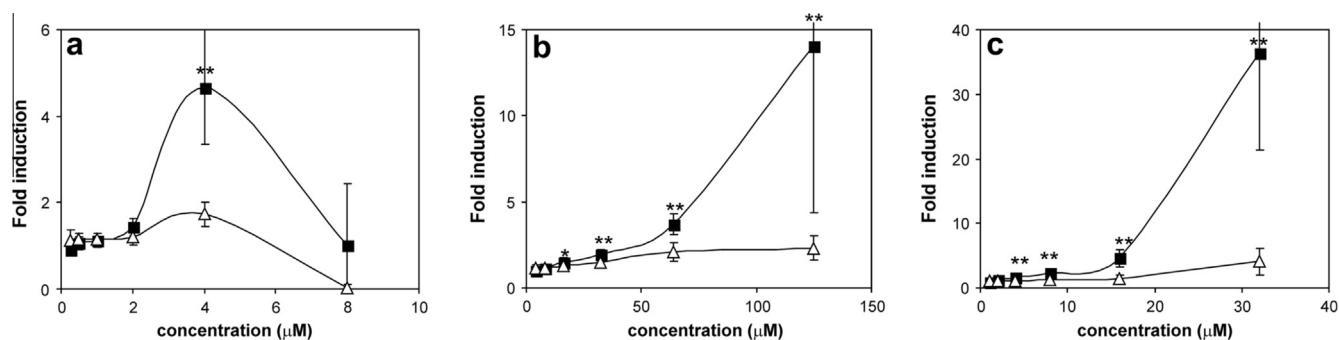


Fig. 1. Luciferase induction after 48 h in the KeratinoSens™ assay for (a) DNCB, (b) cinnamic aldehyde and (c) (2E)-5,6,7-trimethyl-2,5-octadien-4-one. Cells were treated with control siRNA (filled squares) or Nrf2-siRNA (open triangles) three days prior to the start of the experiments. Average values of three independent experiments each performed in triplicate. Different siRNA treatments were compared with Student's *t*-test, asterisk indicates significant at $p < 0.05$, two asterisks indicate significant difference at $p < 0.005$.

is measured 48 h after compound addition) the knockdown was at $64 \pm 13\%$. These results indicate a key role for Nrf2 in the sensitizer-induced luciferase signal.

3.2. The Luciferase signal induced by SDS at cytotoxic conditions and by the false positive Tween80 is also Nrf2-dependent

In order to evaluate the Nrf2-dependence of the false positive outcomes for Tween80 or SDS, the Nrf2-siRNA experiments were also performed with these two chemicals (Fig. 2). Interestingly, similarly to the sensitizers tested above, the dose-dependent upregulation of the luciferase signal for Tween80 was lost in

presence of the Nrf2-siRNA. In addition, SDS only induced a significant luciferase signal at a single cytotoxic concentration, 44 μM with 38% viable cells, confirming previous results. This response due to SDS was completely suppressed by Nrf2-siRNA.

3.3. Induction of prototypic endogenous Nrf2-dependent genes by skin sensitizers parallels induction of the luciferase at RNA level

To better understand whether the luciferase signal truly reflects the sensitizer-induced native Nrf2 response, the cells were exposed to DNCB and cinnamic aldehyde. The sensitizer induced regulation of endogenous, Nrf2-dependent genes (NQO1, HMOX1,

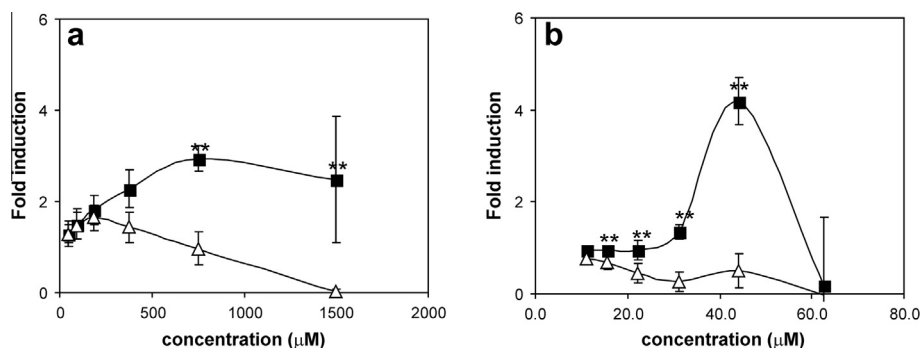


Fig. 2. Luciferase induction after 48 h in the KeratinoSens™ assay for (a) Tween 80 and (b) SDS. Cells were treated with control siRNA (filled squares) or Nrf2-siRNA (open triangles) three days prior to the start of the experiments. Average values of three independent experiments each performed in triplicate. Different siRNA treatments were compared with Student's *t*-test, two asterisks indicate significant difference at $p < 0.005\%$.

AKR1C2) and *Nrf2* was then compared to the luciferase gene. To allow for a direct comparison, the luciferase expression was quantified directly at the mRNA level in these experiments (Fig. 3), and not at the activity level as done in the routine assays. Cinnamic aldehyde and DNCB induced transcription of the luciferase gene *luc2* after both 8 h and 24 h exposure. This induction was very similar to the induction of the endogenous *AKR1C2* gene, whereas a weaker induction was observed for *NQO1*. The most pronounced fold-induction was observed for *HMOX1*, which was induced particularly strongly by cinnamic aldehyde. The induction relative to the solvent control was statistically highly significant for all treatments. The Nrf2-gene itself was not significantly up-regulated by the sensitizers.

3.4. The induction of the endogenous Nrf2-target genes *NQO1* and *AKR1C2* and *luc2* are similarly dependent on Nrf2

We repeated the above experimental series with cinnamic aldehyde to quantify gene expression at the RNA level in presence of Nrf2-siRNA (Fig. 4). A similar induction of *luc2*, *AKR1C2* and *NQO1* was observed as noted above, while the gene induction of these three genes was strongly reduced by Nrf2-siRNA. For *NQO1* and *AKR1C2* the basal mRNA levels were also reduced by Nrf2-siRNA. *HMOX1* was again the gene with the highest fold-induction by cinnamic aldehyde, however this induction was not completely dependent on Nrf2: The fold induction over control was reduced from 26 to 7-fold by the siRNA treatment. The effect of Nrf2-siRNA on the cinnamic aldehyde-induced gene expression was statistically significant at $p < 0.001$ for all five genes. The effect of Nrf2-siRNA on the basal expression (DMSO treatment) of the four

endogenous genes was also statistically significant at $p < 0.001$ and it was significant at $p = 0.01$ for *luc2*.

3.5. Comparison of the Nrf2-dependence for a wider array of sensitizer-induced genes

Next, the induction by DNCB and cinnamic aldehyde of the genes listed in Table 2 and the Nrf2-dependence of this induction was evaluated. Results are shown in Figs. 5–7. Fig. 5 summarizes the results for *Luc2* and the endogenous genes under Nrf2 control. The sensitizer-dependent induction of *AKR1C2*, *Luc2* and *SRXN1* was completely suppressed by the siNrf2-treatment, confirming and extending the previous results. The expression of *HMOX1* was only partly, but significantly, reduced by Nrf2-siRNA for cinnamic aldehyde. In DNCB exposed cells this effect was not seen, confirming that sensitizer induced *HMOX1*-induction may only be partially regulated by Nrf2.

The induction of heat-shock protein related genes could also be verified in the KeratinoSens™ cell line (Fig. 6). Expression proved to be dependent on cell density as the induction was only observed in these experiments conducted at half the cell density as the one used in the standard KeratinoSens™ assay. The DNCB dependent induction of *HSP1A1*, *HSP1B1* and *DNAJB4* was not under control of Nrf2 and in some cases expression of these genes was even slightly enhanced. The induction by cinnamic aldehyde was weaker as compared to DNCB, and a weak reduction due to Nrf2-siRNA was noted at the lower dose and for *HSP1B1* and *DNAJB4* only.

Fig. 7 summarizes the Nrf2-dependence of other sensitizer-specific genes that are related to cellular stress. In general, the sensitizer-induced up-regulation of all these markers could be

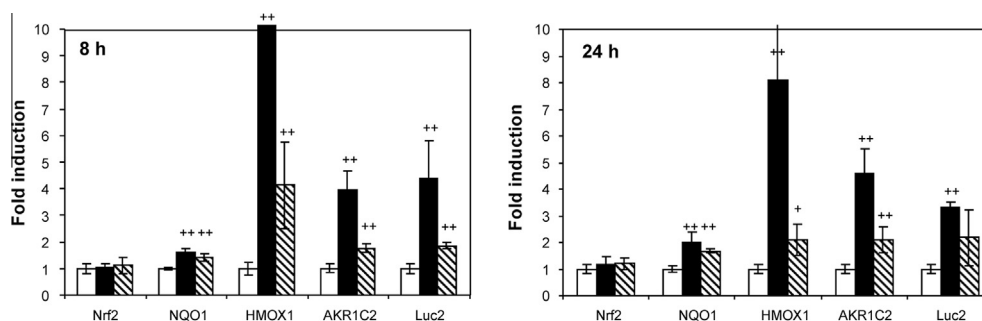


Fig. 3. The induction of Nrf2, *NQO1*, *HMOX1* and *AKR1C2* and of the recombinant luciferase gene *luc2* in the KeratinoSens™ cell line treated for 8 or 24 h with DMSO solvent (open bars), 125 μM cinnamic aldehyde (filled bars) or 8 μM DNCB (striped bars). Average values of two independent experiments each performed in triplicate. Note: *HMOX1* induction by cinnamic aldehyde at 8 h is off-scale and at 36-fold. All data were normalized to the housekeeping gene GAPDH and then for each gene, the data were normalized to the solvent control. Treatments with significant induction over solvent control at $p < 0.001$ are marked with ++; and with + for $p < 0.01$. (*luc2* induction by DNCB at 24 h: $p < 0.05$).

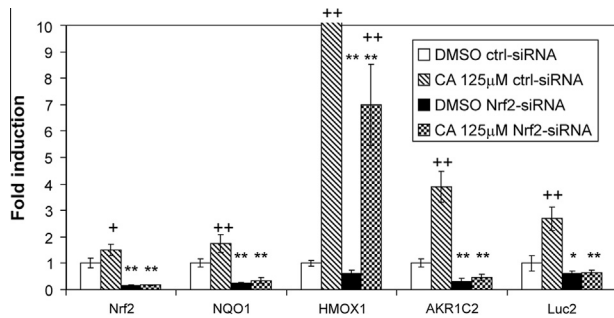


Fig. 4. Induction of Nrf2, NQO1, HMOX1 and AKR1C2 and recombinant luciferase gene *luc2* in the KeratinoSens™ cell line pre-treated with control siRNA or Nrf2-siRNA and then treated for 8 h with cinnamic aldehyde (CA) or DMSO control. Average and standard deviation of results from two independent experiments with each triplicate analysis for each treatment. Note: HMOX1 induction by cinnamic aldehyde with ctrl-siRNA is off-scale and at 26.7-fold. All data were normalized to the housekeeping gene GAPDH and then for each gene, the data were normalized to the solvent control of the ctrl-siRNA treated cells. Each chemical treatment was compared between the fold induction with ctrl and Nrf2-siRNA, and treatments with significant difference at $p < 0.001$ are marked with **; and with * for $P = 0.01$. Treatments with significant induction over solvent control at $p < 0.001$ are marked with ++; and with + for $p < 0.01$. —.

verified in the KeratinoSens™ cell line, although it was not significant at the $p < 0.01$ level in all cases. For all genes the upregulation caused by DNCB was stronger than that caused by cinnamic aldehyde. No dependence on Nrf2 was observed for these genes, with the exception of the induction of *FOSL1* by cinnamic aldehyde.

3.6. Gene induction by the irritant SDS and false negatives in KeratinoSens™

Finally, we tested the induction of the sensitizer-specific markers by SDS as reference irritant and the acyl transfer agents phenyl benzoate and phthalic anhydride, which are false negatives in the KeratinoSens™ assay. Fig. 8 lists the results for Nrf2-dependent genes and heat-shock genes; the induction of all these genes by cinnamic aldehyde reported above was reproduced in this independent set of experiments. Neither phenyl benzoate nor phthalic anhydride induced gene expression, also, SDS did not induce these genes after the 8 h exposure, although the 44 µM dose of SDS did induce the luciferase after 48 h as shown in Fig. 2. This dose is cytotoxic at the 48 h time point but not at the 8 h time point. It is noteworthy that the response of the heat-shock genes and HMOX1 for

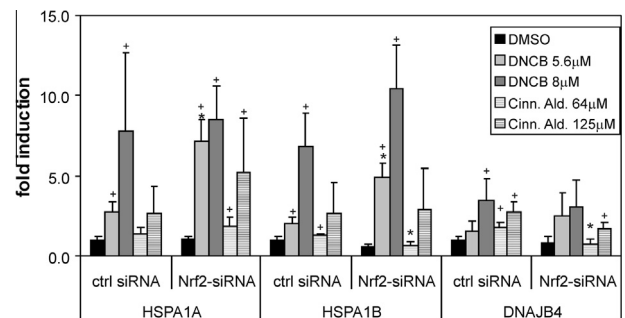


Fig. 6. Induction of heat shock genes HSPA1A, HSPA1B and DNAJB4 in the KeratinoSens™ cell line pre-treated with control siRNA or Nrf2-siRNA, and then treated for 8 h with two doses of DNCB, cinnamic aldehyde or DMSO control. Average and standard deviation of results from two independent experiments with each triplicate analysis for each treatment. Each chemical treatment was compared between the fold induction with ctrl and Nrf2-siRNA, and treatments with significant difference at $p < 0.01$ are marked with *. Treatments with significant induction over solvent control at $p < 0.01$ are marked with +.

these chemical treatments is similar to the Nrf2-regulated genes, although they are not or only partly Nrf2-dependent. The induction of the additional genetic markers is summarized in Fig. 9. The most striking result is the response of *FOS*: This gene, although only moderately up-regulated by cinnamic aldehyde, was strongly up-regulated by SDS, phenyl benzoate and especially by phthalic anhydride. This latter chemical did not significantly induce any of the other markers, while ATF3 and STC2 were also affected by SDS and phenyl benzoate.

4. Discussion

The aim of the current project was to increase the mechanistic understanding of the signal measured in the KeratinoSens™ assay and to explore possibilities to improve the predictivity of the assay with additional sensitizer-specific gene markers. We demonstrate that, after exposure to sensitizers, the induction of endogenous Nrf2-dependent genes and the recombinant *luc2* gene are comparable at the transcription level. This indicates that the *luc2* gene in the KeratinoSens™ cell line is a valid surrogate to the measurement of endogenous Nrf2-dependent genes, such as *AKR1C2*, *NQO1* and *SRXN1*. Furthermore, the sensitizer-induced luciferase signal is strongly dependent on Nrf2 and this shows that the enhancer element in this configuration retains the original specificity for Nrf2.

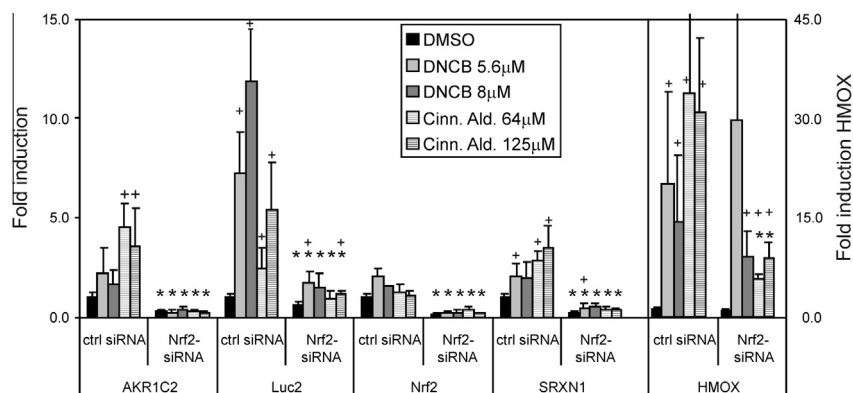


Fig. 5. Induction of Nrf2, AKR1C2, HMOX1 and SRXN1 and the recombinant luciferase gene *luc2* in the KeratinoSens™ cell line pre-treated with control siRNA or Nrf2-siRNA and then treated for 8 h with two doses of DNCB, cinnamic aldehyde or DMSO control. Average and standard deviation of results from two independent experiments with each triplicate analysis for each treatment. Note: different y-axis for HMOX1 on the right side. All data were normalized to the housekeeping gene GAPDH and then for each gene, the data were normalized to the solvent control in ctrl-siRNA treated cells. Each chemical treatment was compared between the fold induction with ctrl and Nrf2-siRNA, and treatments with significant difference at $p < 0.01$ are marked with *. Treatments with significant induction over solvent control at $p < 0.01$ are marked with +.

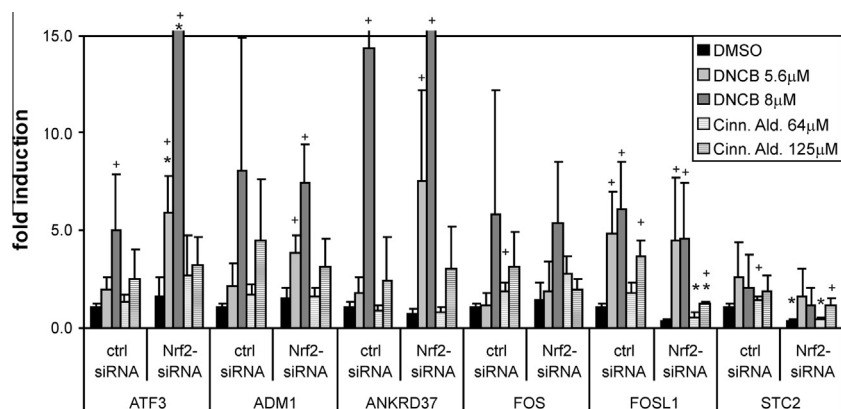


Fig. 7. Induction of emerging genetic markers for skin sensitizers (for reference see Table 1) in the KeratinoSens™ cell line pre-treated with control siRNA or Nrf2-siRNA and then treated for 8 h with two doses of DNCB, cinnamic aldehyde or DMSO control. Average and standard deviation of results from two independent experiments with each triplicate analysis for each treatment. Each chemical treatment was compared between the fold induction with ctrl and Nrf2-siRNA, and treatments with significant difference at $p < 0.01$ are marked with *. Treatments with significant induction over solvent control at $p < 0.01$ are marked with +. Note: the induction by 8 μ M DNCB was off-scale in Nrf2-treated cells for ATF3 (23.1-fold ANKRD37 (15.5-fold).

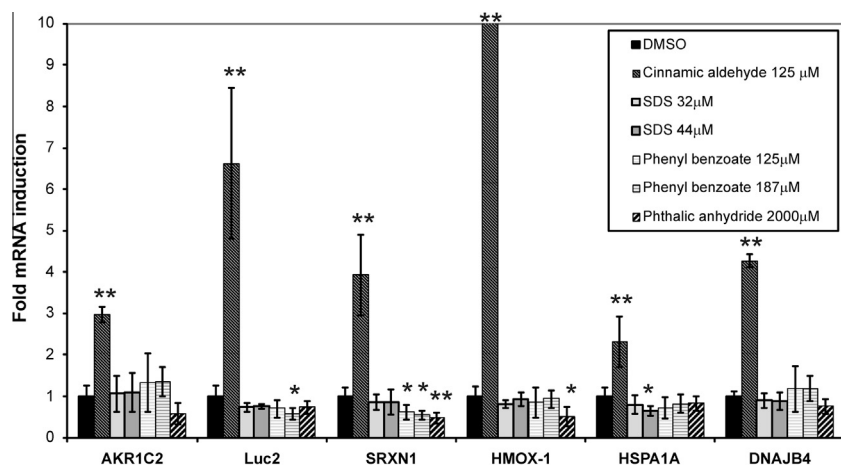


Fig. 8. Induction of endogenous Nrf2 dependent genes and heat-shock genes in KeratinoSens™ cells treated for 8 h with cinnamic aldehyde as positive control, SDS as reference irritant and the acyl transfer agents phenyl benzoate and phthalic anhydride as false negatives in the KeratinoSens™ assay. Average and standard deviation of results from two independent experiments with each triplicate analysis for each treatment. Note: for HMOX1 the induction by cinnamic aldehyde was off-scale and at 25.9-fold. Treatments with significant difference from solvent control at $p < 0.001$ are marked with **; significant differences at $p < 0.01$ are marked with *.

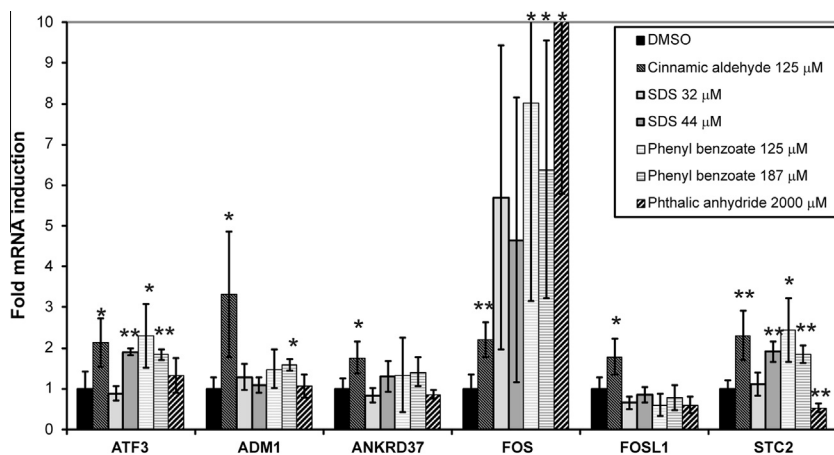


Fig. 9. Induction of miscellaneous genetic markers for sensitizers (for reference see Table 1) in KeratinoSens™ cells treated for 8 h with cinnamic aldehyde as positive control, SDS as reference irritant and the acyl transfer agents phenyl benzoate and phthalic anhydride as false negatives in the KeratinoSens™ assay. Average and standard deviation of results from two independent experiments with each triplicate analysis for each treatment. Note: for FOS the average induction by phthalic anhydride was off-scale and at 12.0-fold. Treatments with significant difference from solvent control at $p < 0.001$ are marked with **; significant differences at $p < 0.01$ are marked with *.

Besides demonstrating the functionality of the Keratinosens™ assay we aimed at gaining insight into the reason why certain compounds, for example Tween 80, are false positive. Furthermore, the irritant SDS induced the luciferase signal as well, but only at a cytotoxic level, demonstrating that including the viability in the prediction model is of importance to maintain the specificity of the assay. Our results indicate that this response elicited by SDS or Tween80 is also Nrf2-dependent. However, both of these chemicals lack the typical electrophilic features of Nrf2-inducing sensitizers. On purely theoretical grounds, there are two possibilities to explain this observation. These chemicals could activate formation of endogenous Nrf2-inducing compounds, such as 4-hydroxynonenal formed by lipid peroxidation or the messenger H₂O₂. Alternatively, these chemicals may trigger a cascade that results in Nrf2-activation, which is not directly related to electrophilic stress. Interestingly, Tween80 was also found false positive in an assay that measures depletion of cell-surface thiols in dendritic cells, a process which is initiated by MAPK p38 activation (Suzuki et al., 2009). Indeed, activation of p38 has been shown to have a positive effect on Nrf2-induction in several studies (Cheng et al., 2010; Gong et al., 2004; Ogborne et al., 2005; Paine et al., 2010; Zhang et al., 2006). However, further work is required to understand if p38 activation is indeed related to Nrf2 activation. A crosstalk between sensitizer-induced kinase activation and Nrf2 activation was recently proposed with activation of PI3K leading to down-stream activation of Nrf2 (McKim et al., 2012).

It was further explored which sensitizer-induced gene markers were under Nrf2 control. *HMOX1*, which is classically considered as a key Nrf2-regulated gene, has been found as the top discriminating gene in HaCaT cells exposed to sensitizers (Vandebriel et al., 2010; van der Veen et al., 2013). It was also up-regulated by sensitizers in dendritic cells (Ade et al., 2009; Arkusz et al., 2010; Johansson et al., 2011). Given that different gene-chip studies often yield very different gene signatures, it is a remarkably stable marker. This highly reproducible and strong induction could also be verified in our study and this confirms the results in wild-type HaCaT (van der Veen et al., 2013). The fact that this gene comes out as a prominent marker from many studies could be due to the extraordinarily high dynamic range (25–30 fold induction by cinnamic aldehyde) which makes it easy to pick up significant changes. Remarkably, *HMOX1* expression was only partly suppressed by Nrf2-knockdown. This observation has not been reported before for skin sensitizers, but it is known that the regulation by Nrf2 through the ARE-enhancer of *HMOX1* is more complex than for other ARE-enhancer genes (MacLeod et al., 2009), as it involves the competitive binding of the repressor BACH1 and the activating transcription factor Nrf2 (Reichard et al., 2007, 2008). One may thus speculate that the sensitizers can simultaneously inactivate BACH1 and activate Nrf2 (as reported for arsenite (Reichard et al., 2008) and this could explain the partial effect seen with Nrf2-knockdown. Alternatively, *HMOX1* may also be induced by sensitizers in an ARE-independent manner, as *HMOX1* is known to contain specific binding sites for AP-1, NF- κ B and probably heat shock factor 1 (Alam and Cook, 2007). Thus, while the *luc2*-response perfectly mirrors other Nrf2-regulated genes, it is unclear whether measuring the *HMOX1*-induction would yield additional information.

A diverse set of emerging markers up-regulated by sensitizers in keratinocytes was also investigated in order to establish whether these were under Nrf2 control. If not, those markers might provide additional non-redundant information that could improve the prediction model. It has recently been shown that the Hsp70 and Hsp40 genes are induced by sensitizers in HaCaT cells and in EpiDerm™ models. It had been proposed that Hsp70 genes could also be regulated by Nrf2, due to the fre-

quently observed parallel induction and the presence of an ARE consensus sequence in the Hsp70-promoter (Almeida et al., 2010). Our data indicate that these genes give indeed a very similar response to the Nrf2-regulated genes (Figs. 6 and 8), but that the induction appears not to be dependent on Nrf2 (Fig. 6). Additionally, other emerging markers appear not to be Nrf2-dependent, but appear to be related to general stress and immune responses. Induction of these genes was in many cases even enhanced in Nrf2-knockdown cells. This is probably due to impaired detoxification in Nrf2-depleted cells. These emerging alternative markers clearly are dependent on alternative sensor mechanisms. One such sensor with a prominent role is the Toll-like receptor (TLR), which is related to the innate immune system and is able to recognize endogenous danger signals and pathogens. The endogenous signals, such as hyaluronic acid, are released by the cells upon stress. The most prominent members of this receptor family are TLR2 and TLR4, whose involvement in skin sensitization has been shown *in vivo* (Martin et al., 2011). The acyl-transfer agent phenyl benzoate is a false negative in the KeratinoSens™ assay. Exposure to this chemical induced the Nrf2-independent gene markers *STC2*, *ATF3* and most prominently *FOS*. Interestingly, *FOS* was the gene with the second highest fold-changes after *HMOX1* in the study by (van der Veen et al., 2013), and this result was confirmed by the data reported in Figs. 7 and 9. This gene was strongly induced by the two acyl-transfer agents, but also by SDS (in the latter case it was significant only at the $p < 0.05$ level). However, of the 10 classical irritants tested in the study of van der Veen, which included Tween 80, only methyl salicylate induced *FOS* (van der Veen et al., 2013). It therefore appears to be a very sensitive marker, but it remains to be verified whether *FOS* is sufficiently specific to be used in a test battery. It has to be stressed that the classification approach of van der Veen et al. is based on a gene signature rather than on the regulation of a single gene. Measurement of these additional markers is clearly not part of the standard KeratinoSens™ assay, but the current data show which of these markers are regulated by a different pathway and might thus be included in the future to add non-redundant information.

In conclusion, the current data prove that the chemical-induced luciferase induction in the KeratinoSens™ cell line is dependent on the Nrf2 activation and that it is a good reflection of the Nrf2 response of endogenous genes. While this is true for the genes *AKR1C2*, *NQO1* and *SRXN1*, only partial dependence on Nrf2 was shown for *HMOX1*. Many other research groups have also described the Nrf2-dependent gene induction by skin sensitizers and various assays have been based on these findings. A more comprehensive screening of the Nrf2-response has been proposed by assessing a number of endogenous Nrf2-regulated genes in parallel. One emerging test in HaCaT keratinocytes measures six Nrf2-dependent genes in parallel (McKim et al., 2010). In addition, a test proposed by ImmunoSearch evaluates the up-regulation of 17 different ARE-dependent genes in parallel (Teissier et al., 2011) in a 3D skin model. In a recently described dendritic cell gene signature (Johansson et al., 2011) the Nrf2-mediated oxidative response is the most highly populated pathway with 10 Nrf2-dependent genes. The dataset of the current work, although limited, indicates that Nrf2-dependent *luc2* induction is a true reflection of the endogenous sensitizer-induced Nrf2-response. Using several Nrf2-dependent assays in a testing strategy would yield redundant information, thereby unnecessarily complicating the evaluation.

5. Funding

No external funding was received for this study.

Conflict of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tiv.2013.09.009>.

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Supplemental table I. RT-PCR primers used in the current study

Gene	Coding for	5'-Primer	3'-Primer	Source
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	Designed with Oligo 4
luc2	Recombinant luciferase in KeratinoSens	TTACCGACGCACATATCGAG	GAAGCTATTCTCGCTGCACA	GenScript Primer Design Tool
AKR1C2	aldo-keto reductase family 1, member C2	Hs_AKR1C2_1_SG	Hs_AKR1C2_1_SG	Qiagen
NQO1	NAD(P)H dehydrogenase (quinone 1)	GCCGCAGACCTTGTGATATTCC	CTCCTATGAACACTCGCTCAAACC	Microsynth
HMOX1	Heme oxygenase 1	CTTGGCTGGCTTCCTTACC	GGCTCCTCCTCCTTTCC	Microsynth
NFE2L1	nuclear factor (erythroid-derived 2)	Hs_NFE2L1_1_SG	Hs_NFE2L1_1_SG	Qiagen
SRXN1	sulfiredoxin 1	Hs_SRXN1_1_SG	Hs_SRXN1_1_SG	Qiagen
HSPA1A	heat shock 70kDa protein 1A	GTGTAACCCCATCATCAGC	AAACAGCAATCTTGGAAGGC	Microsynth
HSPA1B	heat shock 70kDa protein 1B	TCATCAGCGGACTGTACC	GACAAACATACTAAAGAACAAAGG	Microsynth
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	Hs_DNAJB4_2_SG	Hs_DNAJB4_2_SG	Qiagen
ATF3	activating transcription factor 3	Hs_ATF3_1_SG	Hs_ATF3_1_SG	Qiagen
ADM	Adrenomedullin	Hs_ADM_1_SG	Hs_ADM_1_SG	Qiagen
ANKRD37	Ankyrin repeat domain 37	Hs_ANKRD37_1_SG	Hs_ANKRD37_1_SG	Qiagen
FOS	FBJ murine osteosarcoma viral oncogene homolog	Hs_FOS_1_SG	Hs_FOS_1_SG	Qiagen
FOSL1	Fos-like antigen 1	Hs_FOSL1_1_SG	Hs_FOSL1_1_SG	Qiagen
STC2	Stanniocalcin-2	Hs_STC2_1_SG	Hs_STC2_1_SG	Qiagen