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SCHÄFER, Matthias, et al.

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Activation of Nrf2 in keratinocytes causes chloracne (MADISH)-like skin disease in mice

Matthias Schäfer1, 4, Ann-Helen Willrodt1, Svitlana Kurinna1, Andrea S Link2, Hany Farwanah3, Alexandra Geusau4, Florian Gruber4, Olivier Sorg5, Aaron J Huebner6, Dennis R Roop6, Konrad Sandhoff5, Jean-Hilaire Saurat5, Erwin Tschachler4, Marlon R Schneider7, Lutz Langbein8, Wilhelm Bloch9, Hans-Dietmar Beer10 & Sabine Werner3

Abstract

The transcription factor Nrf2 is a key regulator of the cellular stress response, and pharmacological Nrf2 activation is a promising strategy for skin protection and cancer prevention. We show here that prolonged Nrf2 activation in keratinocytes causes sebaceous gland enlargement and seborrhea in mice due to upregulation of the growth factor epigen, which we identified as a novel Nrf2 target. This was accompanied by thickening and hyperkeratosis of hair follicle infundibula. These abnormalities caused dilatation of infundibula, hair loss, and cyst development upon aging. Upregulation of epigen, secretory leukocyte peptidase inhibitor (Slpi), and small proline-rich protein 2d (Sprr2d) in hair follicles was identified as the likely cause of infundibular acanthosis, hyperkeratosis, and cyst formation. These alterations were highly reminiscent to the phenotype of chloracne/metabolizing acquired dioxin-induced skin hamartomas (MADISH) patients. Indeed, SLPI, SPRR2, and epigen were strongly expressed in cysts of MADISH patients and upregulated by dioxin in human keratinocytes in an Nrf2-dependent manner. These results identify novel Nrf2 activities in the pilosebaceous unit and point to a role of Nrf2 in MADISH pathogenesis.

Keywords: acne; sebaceous gland; Nrf2; oxidative stress; skin
Subject Categories: Skin; Metabolism

Introduction

The skin functions as a barrier, which protects our body from harmful environmental insults, including microorganisms, UV light, and toxic chemicals. Many of them lead to the formation of reactive oxygen species (ROS), which at high concentrations damage cellular macromolecules. ROS-induced damage is therefore involved in skin tumor formation, aging, and in the pathogenesis of inflammatory skin diseases, such as psoriasis, atopic dermatitis, and contact dermatitis.

The skin has evolved effective mechanisms for the protection from ROS damage, including ROS detoxification and DNA repair. A master regulator of the cellular antioxidant defense is nuclear factor erythroid derived 2, like 2 (Nrf2). This transcription factor induces expression of various genes involved in the cellular redox balance, including the genes encoding glutamate cysteine ligase modifier (Gclm) and catalytic (Gclc) subunits, phase II detoxifying enzymes, including NAD(P)H dehydrogenase, quinone 1 (Nqo1), and transporters, including multidrug resistance proteins (Mrps).

Nrf2 expression and activity form a basal to suprabasal gradient in the murine epidermis, which contributes to an intra-epidermal cytoprotection gradient. This results in strong protection of the uppermost epidermal layers from ROS-induced damage (Schäfer et al., 2010; Piao et al., 2012). Nrf2 gets further activated by electrophilic compounds, which directly bind and inactivate the Nrf2 inhibitor protein Keap1 (Tong et al., 2006).

Due to its central role in ROS detoxification, Nrf2 is an attractive target for pharmacological protection of the skin. A variety of Nrf2 activators, including sulforaphane, tert-butyl-hydroquinone (tBHQ), and resveratrol, have been discovered, which are antioxidant supplements of cosmetic products. Some of these compounds protected cultured keratinocytes from damage induced by UV irradiation or treatment with...
Nrf2 activation in keratinocytes causes sebaceous gland hypertrophy and sebum congestion

We previously generated transgenic mice expressing a caNrf2 mutant under control of a β-actin promoter and CMV enhancer in keratinocytes using a strategy that allows expression of the transgene in the presence of Cre recombinase. For expression of caNrf2 in all keratinocytes, mice expressing Cre under the control of the keratin 5 (K5) promoter were used (Ramírez et al., 2004). The double transgenic mice, designated K5cre-CMVcaNrf2 mice, are characterized by acanthosis and severe hyperkeratosis in the epidermis (Schäfer et al., 2012). Interestingly, histological analyses and immunofluorescence staining for perilipin 2 (adipophilin, Adph) showed that the sebaceous glands (SG) in back and tail skin (Fig 1A,B) and the meibomian glands of the eyelids (Supplementary Fig 1A) are strongly enlarged in K5cre-CMVcaNrf2 mice. Consistent with these findings, their tail skin was oily and sticky, and the eyes were wet and sometimes partially closed, suggesting sebum/meibum overproduction (seborrhea).

Repeated topical treatment with the Nrf2-activating compounds sulforaphane or tert-butyl hydroquinone (tBHQ) (Schäfer et al., 2012) also caused enlargement of SGs in wild-type mice (Fig 1C), but not in Nrf2 ko mice (Supplementary Fig 1B), demonstrating that activation of endogenous Nrf2 has a similar effect as the caNrf2 transgene.

In addition to the SG abnormalities, K5cre-CMVcaNrf2 mice had dilated hair follicle infundibula (Fig 1D, upper and lower panel). Oil Red O staining revealed the presence of sebum in the widened infundibula of K5cre-CMVcaNrf2 mice (Fig 1D, upper panel), which were characterized by strong acanthosis and hyperkeratosis (Fig 1D, lower panel and 1E). Together, hyperkeratosis of infundibula and the interfollicular epidermis subsequently form a mechanical barrier, which prevent sebum outflow. The dilatation of the infundibulum was even more severe in hair follicles without a hair, most likely because the hair prevented a complete occlusion of the hair follicle by the hyperkeratotic stratum corneum and thus enabled sebum outflow (Fig 1F). Hyperkeratosis, in combination with sebum congestion, lead to keratin and sebum accumulation, and the resulting pressure finally caused dilation of hair follicle infundibula (see scheme in Fig 2F).

K5cre-CMVcaNrf2 mice exhibit cyclic hair loss and malformed hairs

In addition to the SG abnormalities, K5cre-CMVcaNrf2 mice exhibited cyclic hair loss, resulting in patchy baldness during catagen and telogen (Fig 2A and Supplementary Fig 1C–E). This was not due to a reduction in the number of hair follicles in the affected areas (Supplementary Fig 1F). Moreover, the follicles underwent all phases of the hair cycle, although the hair cycle of transgenic mice preceded the cycle of control mice by approximately 4 days (Supplementary Fig 2). Anagen hair follicles had a normal length, and follicles included a hair. Immunostaining of keratins expressed in various root sheath compartments revealed no obvious defects in differentiation of lower hair follicle keratinocytes (root sheath) and trichocytes (hair fiber) during hair formation (Supplementary Fig 3). Thus, hair follicles of K5cre-CMVcaNrf2 mice undergo normal differentiation and obviously form a functional hair during anagen, which, however, is lost prematurely during catagen or telogen. Interestingly, telogen pelage hairs were only anchored in the lower part of hair follicles due to dilution of infundibula (Fig 2B), suggesting that the hair loss during catagen and telogen is the consequence of reduced anchorage of pelage hairs.

The remaining pelage hair as well as the whiskers of K5cre-CMVcaNrf2 mice appeared shaggy and wavy (Supplementary Fig 1G). Furthermore, the majority of pelage hairs was thinner and curly (Supplementary Fig 1H) and had spiky cuticle cells (Supplementary Fig 1J), which explains the shaggy appearance of the remaining fur. These malformations of the remaining hairs are most likely the consequence of the narrowing of the hair canal due to infundibular hyperkeratosis and acanthosis.
Nrf2 activation affects hair follicles and sebaceous glands in a dose-dependent manner

In K5cre-caNrf2 mice, caNrf2 is also under control of a β-actin promoter, but not of a CMV enhancer, resulting in a weaker activation of Nrf2 target genes compared with K5cre-CMVcaNrf2 mice (Scha¨fer et al, 2010). K5cre-caNrf2 mice also had curly hairs and they lost pelage hairs, but this was only observed 5–6 weeks after birth and was restricted to the tail (Supplementary Fig 4A–C). This locally restricted phenotype correlates with the stronger expression of Nrf2 (endogenous Nrf2 plus transgene-derived caNrf2) and its target genes Nqo1, Gclc, Gclm, and sulfiredoxin1 (Srxn1) in the tail compared with the back skin (Supplementary Fig 4D). Histological analyses and Adph immunofluorescence staining of tail skin sections identified enlarged SGs, an increased number of sebocytes (Supplementary Fig 4E–H), sebum-filled infundibula (Supplementary Fig 4E arrow), and dilatation of infundibula (Supplementary Fig 4F). These abnormalities were similar as in K5cre-CMVcaNrf2 mice, but less pronounced, demonstrating a strong dependency of the phenotype on the level of Nrf2 activation.

Aged K5cre-CMVcaNrf2 mice develop large cutaneous and meibomian cysts

In one-year-old (1 y) K5cre-CMVcaNrf2 mice, we found a more severe dilatation and keratinization of infundibula in back (Supplementary Fig 5A) and tail skin (Fig 2C) when compared to young mice. In addition, keratinized infundibular cysts of different sizes (Fig 2C) and a few macroscopically visible large cysts were observed in the tail skin (Fig 2D, Supplementary Table 1). The
Cutaneous cysts were mainly filled with keratin and to a much lesser extent with lipids as indicated by H&E and oil red O staining (Fig 2C,E). In addition, serial sections revealed that only small or no SGs are associated with the large cysts. In some of the large cysts, we found a rupture of the epithelial wall, most likely due to keratin overproduction and consequent enlargement of the cyst lumen (Supplementary Fig SB). These cysts are of hair follicle origin, since the epithelial cells expressed K14 (ORS- and basal infundibulum cells), keratin K6 (ORS- and active/hyperproliferative infundibulum cells), but not K10 and loricrin (Lor) (both missing in ORS cells).
Thus, in aged K5cre-CMVcaNrf2 mice, the continuous hyperkeratinization of infundibula, which starts already in young mice, leads to progressive dilatation of infundibula and ultimately to formation of cutaneous cysts (Fig 2F).

In preputial glands of aged K5cre-CMVcaNrf2 mice, where caNrf2 is also strongly expressed as reflected by the strong expression of its target genes (Supplementary Fig 5D), we observed hyperkeratosis of the luminal duct (Supplementary Fig 5E). Ultimately, this resulted in preputial gland swelling due to sebum congestion (Supplementary Fig 5F,G).

**Activation of Nrf2 induces sebocyte hyperproliferation and disturbs sebocyte differentiation and lipid metabolism**

In the back of P32 K5cre-CMVcaNrf2 mice as well as in the tail of K5cre-caNrf2 mice, we found a significant increase in the number of...
proliferating sebocytes in relation to the circumference of the SG as determined by 5-bromo-2′-deoxyuridine (BrdU) labeling (Fig 3A,B and Supplementary Fig 6A,B). This provides a likely explanation for the severe SG hyperplasia in these mice. Furthermore, expression of the SG markers Adph and peroxisome proliferator-activated receptor gamma (Pparg) (Rosenfield et al., 1999) (Fig 3C) was increased, which correlates with the enlargement of SG in these mice. However, the late SG differentiation marker melanocortin-5 receptor (Mc5r) (Chen et al., 1997; van der Kraan et al., 1998) was not upregulated concomitantly (Fig 3C), indicating a disturbance in late differentiation or maturation of sebocytes. Consistent with this observation, we found a differential expression of stearoyl-CoA desaturases (Scds; Δ⁹ desaturases), which catalyze the conversion of saturated into mono-unsaturated fatty acids (Guillou et al., 2010). Scd1 was strongly upregulated, while Scd3 was downregulated in the skin of K5cre-CMVcaNrf2 mice (Fig 3D). Similar alterations in the expression of these genes were also detected in wild-type mice upon activation of endogenous Nrf2 by repeated TBOH treatment (Supplementary Fig 6D,E). However, expression of Adph, Pparg, and Scd1 were not significantly upregulated at P2.5 in K5cre-CMVcaNrf2 mice (Fig 3E), suggesting that the regulation of these genes is not a direct consequence of Nrf2 activation, but rather a consequence of the increase in sebocyte proliferation and abnormalities in sebocyte differentiation.

High-performance thin-layer chromatography of the back skin lipids of K5cre-CMVcaNrf2 mice revealed a 27% reduction in triacylglycerides, while the levels of fatty acids, cholesterol, and cholesterol esters were normal (Fig 3F,G). These alterations in sebocyte lipid metabolism may involve the deregulation of Scds in K5cre-CMVcaNrf2 mice.

Nrf2 activation induces hyperproliferation of sebocytes by upregulation of epigen

To determine the molecular mechanisms underlying the sebocyte phenotype, we analyzed microarray data from skin of P2.5 control and K5cre-CMVcaNrf2 mice (Schäfer et al., 2012). Interestingly, this analysis revealed upregulation of the epidermal growth factor (EGF) family member epigen (Epgn), which had previously been shown to induce sebocyte enlargement when overexpressed in transgenic mice (Dahlhoff et al., 2009). In situ hybridization revealed strong Epgn expression in the interfollicular epidermis and the pilosebaceous unit, including SGs, in control and in particular in K5cre-CMVcaNrf2 mice (Fig 3H, Supplementary Fig 6F representing different staining intensities). Furthermore, qRT-PCR analysis confirmed upregulation of Epgn in the back skin of P2.5 and P32 K5cre-CMVcaNrf2 mice (Fig 3I) and in their preputial glands (Supplementary Fig 6G), as well as in the tail skin of K5cre-caNrf2 mice (Supplementary Fig 6C). We also found increased expression of other EGF family members, including amphiregulin (Areg), betacellulin (Btc), epiiregulin (Ereg), and heparin-binding EGF-like growth factor (Hbegf) in the skin of P32 (Fig 3J), but not of P2.5 K5cre-CMVcaNrf2 mice.

Inhibition of EGFR signaling by systemic treatment of control and K5cre-CMVcaNrf2 mice with the EGFR kinase inhibitor Gefitinib (Iressa) resulted in a 25% reduction in SG area of control mice compared with vehicle-treated mice, and an even stronger reduction (33%) in K5cre-CMVcaNrf2 mice (Fig 4A). These results suggest that the hyperproliferation and hyperplasia of the SG are the consequence of elevated expression of EGF family members, followed by enhanced EGFR signaling. Epgn seems to be the initial growth stimulus, since it was upregulated in P2.5 K5cre-CMVcaNrf2 mice and thus prior to the development of the SG phenotype. Consistent with this hypothesis, transgenic mice expressing human EPGN under control of a CMV enhancer and β-actin promoter also showed enhanced sebocyte proliferation and severe SG hyperplasia (Dahlhoff et al., 2009). Moreover, the regulation of Adph, Pparg Mc5r, and Scd1, 2, 3, and 4 was similar in EPGN transgenic mice and K5cre-CMVcaNrf2 mice (Fig 4B,C; see Fig 3C,D).

Epigen is a direct target of Nrf2, which is upregulated upon Nrf2 activation

Upregulation of Epgn expression was observed in primary keratinocytes from K5cre-CMVcaNrf2 mice (Fig 4D) as well as in sulfone-treated keratinocytes from wild-type mice, but not from Nrf2ko mice (Fig 4E). Thus, it occurs in a cell-autonomous and Nrf2-dependent manner, suggesting that Epgn is a direct Nrf2 target gene. This hypothesis is supported by in silico identification of a classical antioxidant response element (ARE; Nrf2 binding site) (Rushmore et al., 1991) in the murine Epgn gene promoter, 4.8 kb upstream of the transcription start site (Fig 4F). Chromatin immunoprecipitation (ChIP) using a mixture of lysates from back and tail epidermis of wild-type mice using an Nrf2 antibody revealed binding of endogenous Nrf2 to the Epgn ARE, but not to a different promoter region (Fig 4G). ChIP analysis of lysates using a histone H3 dimethyl Lys4 (H3K4me2) antibody, a marker of transcriptionally active chromatin (Pekowska et al., 2011), showed no increase in histone H3 dimethylation at the Epgn ARE compared with a non-coding DNA region (ncDNA) in control mice (Fig 4H). This suggests that Epgn gene expression is not activated by endogenous Nrf2 under homeostatic conditions. However, there was an increase in histone H3 dimethylation at the Epgn ARE in K5cre-CMVcaNrf2 mice (Fig 4I), indicating that caNrf2 activates Epgn transcription via binding to the identified ARE. Taken together, these data reveal that Nrf2 binds to the Epgn ARE and that Nrf2 activation enhances Epgn gene transcription. This leads to increased EGFR signaling and concomitant induction of sebocyte proliferation, resulting in sebaceous gland hyperplasia (Fig 4J).

Acanthosis and hyperkeratosis of infundibula in K5cre-CMVcaNrf2 mice are most likely the consequence of Slpi, Sprr2d, and Epgn upregulation

BrdU labeling showed that thickening of hair follicle infundibula results from increased proliferation of keratinocytes (Fig 5A). Gefitinib treatment caused a minor reduction in the thickness of infundibula (Fig 5B), but a significant reduction in the proliferation rate of keratinocytes compared with vehicle-treated mice (Fig 5C). Thus, activation of EGFR signaling obviously enhances keratinocyte proliferation and hyperplasia of infundibula in K5cre-CMVcaNrf2 mice. In the epidermis, Nrf2-mediated upregulation of Sprr2d reduced the mechanical stability of the corneocytes, resulting in an impairment of epidermal barrier function (Schäfer et al., 2012). This deficiency caused mild inflammation and upregulation of keratinocyte mitogens, resulting in keratinocyte hyperproliferation. We also found upregulation of Sprr2 expression in differentiated keratinocytes of infundibula (Fig 5D upper panel) and a clustering of mast...
cells around the hair follicles of K5cre-CMVcaNrf2 mice (Fig 5D middle panel). This result strongly suggests that Sprr2d upregulation causes acanthosis of infundibula by reducing barrier functionality

Slpi, which inhibits kallikrein 7, a protease required for corneodesmosome cleavage (Franzke et al., 1996), was significantly upregulated in the epidermis of K5cre-CMVcaNrf2 transgenic mice, leading to reduced desquamation and consequently hyperkeratosis (Schaef et al., 2012). We observed a significant overexpression of Slpi in the IFN and infundibular stratum corneum (Fig 5D lower panel) and impaired corneodesmosome degradation in the IFN (Fig 5E). Thus, follicular hyperkeratosis in K5cre-CMVcaNrf2 mice most likely results from increased expression of Slpi, which inhibits corneocyte desquamation (Fig 5F).

Interestingly, Sprr2d and Slpi were both upregulated in preputial glands of K5cre-CMVcaNrf2 mice, which became hyperkeratotic upon aging (Supporting Information Fig 6G), suggesting a similar mechanism of action of activated Nrf2 in skin and preputial glands.

Cyst formation in K5cre-CMVcaNrf2 mice is the consequence of continuous follicular hyperkeratosis

K5cre-CMVcaNrf2 mice had a milder epidermal phenotype at 6 m compared with P32 due to partial silencing of caNrf2 expression and

Figure 4. Regulation of Epgn by Nrf2.
A Average SG area of P32 tg/wt and tg/tg mice injected with vehicle or Gefitinib. Note reduction in SG area upon Gefitinib treatment of tg/wt mice (N = 6/5, **P = 0.0025) and tg/tg mice (N = 4, *P = 0.0286). B, C qRT-PCR of Adph, Pparg, Mc5r (B), and Scd1, 2, 3, 4 (C) relative to Gapdh using RNA of back skin from wt and EPGN-tg mice (N = 3). D, E qRT-PCR of Epgn using RNAs of murine primary keratinocytes of wt and Nrf2ko mice treated with vehicle or sulforaphane (E) (N = 4, *P = 0.0286). F Localization of PCR-amplified non-specific region (ns), promoter region (prom), and antioxidant response elements (AREs) in the murine Nqo1 and Epgn genes. G Chromatin immunoprecipitation (ChIP) using lysates from back and tail skin of control mice. Note the enhanced binding of Nrf2 to the Epgn ARE compared with the ns region (Nqo1ns, N = 6, Nqo1ARE, N = 6, *P = 0.0045; Epgn ARE, N = 4, *P = 0.012). H, I ChIP using back and tail skin lysates of wt and Nrf2tg mice (I) with an H3K4me2 antibody. Regions void of annotated ORFs (non-coding, nc DNA), Epgn prom, and Epgn ARE were amplified by qRT-PCR. Note the increase in histone H3 dimethylation at the Epgn ARE compared with ncDNA in wt and Nrf2ko mice treated with vehicle or sulforaphane (E).

J Working model: Nrf2 activation leads to increased Epgn expression, which activates EGFR signaling. This enhances proliferation of sebocyte progenitors, leading to sebaceous gland hyperplasia.

Data information: Values are shown as the mean with s.d. All P-values were calculated by Mann–Whitney U-test.
consequent downregulation of Nrf2 target genes (Schäfer et al., 2012). At the age of 1 year, they showed an even further decrease in expression of the classical Nrf2 target genes Nqo1 (Fig 6A) and Srxn1 (Fig 6B). Expression of Epgn had significantly declined at 6 m (Fig 6C), providing a likely explanation for the low sebum content in the cyst lumen (see Fig 2E) and the reduced size or even absence of SGs associated with large cysts. The decline in Sprr2d (Fig 6D) and Slpi (Fig 6E) expression upon aging of K5cre-CMVcaNrf2 mice, however, was less pronounced, and in particular Slpi mRNA levels were still more than 20-fold higher than in control animals at the age of 1 year (Fig 6E). Consistently, immunohistochemistry revealed strong staining for Slpi in the epithelium and keratin-filled lumen of the cysts of aged K5cre-CMVcaNrf2 mice (Fig 6F). Thus, continuous high expression of Slpi and a consequent reduction of corneocyte desquamation seem to underlie the development of keratinized cysts in aged mice. These findings suggest that differences in the ratio of individual Nrf2 targets result in different cutaneous abnormalities.

Figure 5. Sprr2d and Slpi expression in infundibula of K5cre-CMVcaNrf2 mice.
A. Average number of BrdU-positive cells in infundibula (INF) of tg/wt and tg/tg mice (N = 4).
B,C. Average thickness of infundibula and number of PCNA-positive cells in infundibula of P32 tg/wt and tg/tg mice injected with vehicle or Gefitinib (N = 4/5).
D. Upper panel: immunohistochemistry of Sprr2 on longitudinal P10 tg/wt and tg/tg back skin sections. Note staining of Sprr2 in differentiated infundibular keratinocytes in tg/tg mice. Scale bar: 25 μm. Middle panel: toluidine blue staining for detection of mast cells on longitudinal back skin sections of P32 tg/wt and tg/tg mice. Note increase in number of mast cells in tg/tg mice and assembly of mast cells along the interfollicular epidermis and upper part of the hair follicles. Scale bar: 100 μm. Lower panel: immunohistochemistry of Slpi on longitudinal P10 tg/wt and tg/tg back skin sections. Inset in lower panel shows immunohistochemistry without primary antibody. The dashed line marks the basement membrane of the interfollicular epidermis. Note staining of Slpi (indicated by arrow) in differentiated infundibular keratinocytes and stratum corneum in tg/tg mice. Scale bar: 25 μm.
E. Electron microscopy of infundibular stratum corneum of P32 tg/wt and tg/tg mice. Corneocyte layers in lower panel were numbered from basal to distal (C1–C3). Arrows point to corneodesmosomes. Note delayed corneodesmosome degradation in tg/tg mice. C. corneocyte.
F. Working model: Nrf2 activation leads to upregulation of Slpi, Sprr2d, and Epgn in hair follicle infundibula. Slpi upregulation promotes inhibition of CE protease activity. Consequently, corneodesmosome cleavage is reduced, leading to decreased desquamation and thereby to hyperkeratosis of infundibula. Sprr2d upregulation reduces epidermal barrier functionality, resulting in increased inflammation and consequently stimulation of proliferation. Epgn and other epidermal growth factor (EGF) family members stimulate proliferation of infundibular keratinocytes via EGFR signaling. Together, this results in acanthosis of hair follicle infundibula.

Data information: Values are shown as the mean with s.d.
SPRR2 and SLPI are strongly expressed in cysts of MADISH patients

The histopathological features of K5cre-CMVcaNrf2 mice showed remarkable similarities to the cutaneous abnormalities seen in patients with chloracne/MADISH (Saurat & Sorg, 2010), including hair follicle proliferation, hyperplasia, and hyperkeratosis as well as formation of keratinized cysts with small or no SGs (Fig 7A) (Panteleyev & Bickers, 2006). This prompted us to investigate the potential involvement of similar pathomechanisms in caNrf2-transgenic mice and MADISH patients. Indeed, we detected strong expression of SPRR2, SLPI, EPGN and of the classical NRF2 target NQO1 in the epidermis and cyst epithelium of MADISH patients, with particularly strong staining of SPRR2, SLPI, and NQO1 in differentiated keratinocytes (Fig 7B–E). Since all these genes are concomitantly activated by caNrf2 in mouse keratinocytes in vivo, it seems most likely that NRF2 is activated in keratinocytes of MADISH patients. The consequent upregulation of SLPI, SPRR2, and EPGN could then contribute to cyst formation in the skin of MADISH patients by the observed interference with stratum corneum desquamation and epidermal barrier formation.

Dioxin treatment enhances expression of SLPI, SPRR2D, and EPGN in human keratinocytes in an Nrf2-dependent manner

The pathology of MADISH patients develops after intoxication with halogenated aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Geusau et al, 2001; Sorg et al, 2009; Saurat et al, 2012). Interestingly, we found upregulation of SLPI, SPRR2D, and EPGN in cultured human foreskin keratinocytes (HFKs) by treatment with TCDD (Fig 8A). TCDD is known to activate the aryl hydrocarbon receptor (AHR), and this was verified by the strong increase in the expression of cytochrome P450 1A1 and 1B1 (CYP1A1 and CYP1B1) (Fig 8B), which are major AHR target genes (Hayes et al, 2009). Concomitantly, expression of NRF2 and NQO1 was induced (Fig 8C). This finding is consistent with previous observations showing that TCDD via AHR leads to increased expression and/or activation of NRF2 in different cell types (Hayes et al, 2009). We next transfected HFKs with two different siRNAs targeting NRF2 mRNA (Supplementary Fig 7A). Interestingly, the basal and TCDD-induced expression of NQO1, SLPI, SPRR2D, and EPGN was strongly diminished after transfection with NRF2 siRNA (Fig 8D). Furthermore, transfection with two different siRNAs targeting AHR (Supplementary Fig 7B) resulted in a strong reduction of the TCDD-induced expression of SLPI, SPRR2D, and EPGN (Fig 8E). These data provide evidence for a TCDD-induced activation of an AHR-NRF2 axis in MADISH patients, which results in upregulation of the NRF2 targets SLPI, SPRR2D, and EPGN with subsequent development of cystic lesions.

Discussion

Nrf2-activating compounds are used for skin protection under stress conditions as well as for cancer prevention. Unfortunately, there is accumulating evidence for severe adverse effects of Nrf2 activation.

Figure 6. Differential regulation of Nrf2 target genes in K5cre-CMVcaNrf2 mice.

A–E qRT-PCR for Nqo1, Srxn1, Epgn, Sprr2d, and Slpi relative to Gapdh using RNAs from skin of P2.5, P32, 6-m- and 1-y-old tg/wt and tg/tg mice (N = 3/4). Expression in tg/wt mice was arbitrarily set as 1.

F Immunohistochemistry of Slpi on 1 y tg/wt and tg/tg back skin sections. Note strong staining of Slpi in cyst epithelium and keratinized lumen compared with differentiated keratinocytes of the epidermis (indicated by arrow). Scale bar: 100 μm.

Data information: Values are shown as the mean with s.d.
In this study, we identified unexpected abnormalities in the pilosebaceous unit upon long-term activation of Nrf2 in the skin. The phenotype was dependent on the level and duration of Nrf2 activation, which resulted in different ratios of Nrf2 targets. This needs to be considered when Nrf2-activating compounds are used for skin protection in vivo.

We identified acanthosis and hyperkeratosis of infundibula and the interfollicular epidermis as the reason for dilatation of infundibula in caNrf2-transgenic mice. These alterations prevent sebum outflow due to narrowing and plugging of the hair canal. An increase in sebum production by the enlarged SG further aggravates sebum congestion. The dilatation of infundibula, in turn, reduces
the anchorage of hairs, providing a likely explanation for their premature loss during late catagen and telogen. By contrast, we excluded alterations in hair follicle morphogenesis or cycling or in the differentiation of hair follicle keratinocytes. The follicular and epidermal hyperkeratosis and acanthosis also explain the thinning and malformation of hairs, because they prevent a proper outgrowth of newly forming hairs during anagen. The hair loss also promotes the occlusion of the hair canal and thereby sebum congestion and dilatation of infundibula.

Several results of this study strongly suggest that Nrf2-induced upregulation of Epgn is the primary reason for the observed SG abnormalities and the acanthosis of infundibula: (i) Epgn was upregulated in K5cre-CMVcaNrf2 mice prior to the development of histological abnormalities, and it was strongly expressed in the pilosebaceous unit, including the SGs of adult K5cre-CMVcaNrf2 mice; (ii) EPGN transgenic mice showed similar expression profile of genes involved in sebocyte differentiation and lipid metabolism (this study); (iii) pharmacological blockade of EGFR signaling reduced hyperplasia of SGs and proliferation of infundibula in K5cre-CMVcaNrf2 mice; (iv) Epgn upregulation occurred in a cell-autonomous and Nrf2-dependent manner in cultured keratinocytes; and (v) ChIP experiments revealed that Nrf2 binds to a distal ARE in the Epgn promoter region and that Nrf2 activation causes transcriptional activation of Epgn.

In addition to this direct effect, acanthosis of infundibula in K5cre-CMVcaNrf2 mice may result at least in part from upregulation of Sprr2d and subsequent disruption of the follicular barrier. This allows the penetration of allergens and/or bacteria from the lumen of hair follicles, which are colonized by commensal microbes (Nakatsuji et al., 2013). The resulting accumulation of immune cells stimulates proliferation of keratinocytes in a paracrine manner through upregulation of keratinocyte mitogens as previously demonstrated for epidermal keratinocytes (Schäfer et al., 2012). The infundibular
hyperkeratosis and impaired stratum corneum desquamation can be explained by overexpression of Slpi, a direct target of Nrf2 (Iizuka et al., 2005), in the infundibula of K5cre-CMVcaNrf2 mice. The previously demonstrated inhibition of kallikrein 7 activity by Slpi (Franzke et al., 1996) likely inhibits cornodesmosome cleavage in the infundibular stratum corneum, resulting in impaired desquamation and ultimately follicular hyperkeratosis. Taken together, these results reveal previously unrecognized functions of Slpi, Sprr2d, and Epgn in the pilosebaceous unit.

In aged K5cre-CMVcaNrf2 mice, we observed large keratin-filled cysts, in particular in the tail. The different histopathological abnormalities in aged versus young mice may be due to different ratios in the expression levels of individual Nrf2 target genes. In general, there was an age-dependent decline in the expression of Nrf2 target genes in K5cre-CMVcaNrf2 mice. Epgn expression showed a peak at P32, but was strongly reduced already in 6-month-old mice. This provides a likely explanation for the marginal sebum in the cyst lumen and the reduced size or lack of SG associated with cysts in 1-year-old K5cre-CMVcaNrf2 mice. By contrast, the age-dependent decline in Sprr2d and in Slpi expression was less pronounced. The continuously high expression of these proteins is likely to be responsible for the progressive hyperkeratinization of infundibula and formation of keratinized cutaneous cysts upon aging.

Follicular hyperkeratosis and acanthosis together with SG hyperplasia and seborrhea as seen in young K5cre-CMVcaNrf2 mice are important characteristics of acne in humans. However, the development of Acne Vulgaris also involves Propionibacterium (P.) acnes overgrowth, which only affects humans, but not mice, and which triggers a severe inflammatory response (Makrantonaki et al., 2011). K5cre-CMVcaNrf2 transgenic mice only had mild inflammation, presumably due to an epidermal barrier defect (Schafer et al., 2012), but not as a result of P. acnes overgrowth. Therefore, the phenotype of caNrf2 mice rather resembles the abnormalities seen in human non-inflammatory acne, in particular Acne Comedonica.

The keratinization of the cyst lumen and reduction or lack of SGs in aged mice, in contrast, are remarkably reminiscent to the pathology that develops in MADISH patients (Panteleev & Bickers, 2006; Saurat et al., 2012). Indeed, we found strong expression of NQO1, Slpi, Sprr2d, and Epgn in the affected epidermis and cyst epithelium of these patients. The nuclear staining of SPRR2 in the human foreskin keratinocytes, and TCDD-mediated upregulation of SLPI, SPRR2D, and SLPI was observed in both approaches. Furthermore, NQO1 was strongly expressed in the cyst epithelium of MADISH patients. This suggests that SLPI, SPRR2D, and SLPI are also upregulated by TCDD-mediated activation of AHR and Nrf2 in vivo under these conditions. By contrast, upregulation of these genes is not a general response of hyperproliferative keratinocytes, since it was not observed in human basal cell carcinomas (Supplementary Fig 7C).

Although TCDD induced the expression of NQO1 and the novel Nrf2 targets EPGN, SPRR2D, and SLPI in cultured human keratinocytes in an Nrf2-dependent manner, no significant upregulation of various other classical Nrf2 target genes was detected. It may well be that the rather weak activation of Nrf2 by TCDD is insufficient to induce the expression of most classical Nrf2 target genes, with the exception of NQO1, which is particularly sensitive to Nrf2 activation. TCDD-mediated induction of SPRR2D, SLPI, and EPGN may be achieved by a combination of Nrf2 and AHR that is more potent than Nrf2-mediated activation alone. This novel Nrf2-AHR cross-talk needs further investigations at the molecular level. Independent of the regulatory mechanisms, our data provide strong evidence for a potent function of the newly discovered AHR-NRF2-SLPI/SPRR2D/EPGN axis in the pathogenesis of MADISH. Since this axis is also activated in K5cre-CMVcaNrf2 mice, these animals provide an interesting novel model to study this important toxin-induced disorder.

Materials and Methods

RNA isolation and qRT-PCR

Total cellular RNA was isolated as previously described (Chomczynski & Sacchi, 1987; Werner et al., 1993) and further purified using the Nucleospin RNAII kit (Macherey-Nagel, Düren, Germany) or the MiniElute kit (Qiagen, Hilden, Germany). cDNA synthesis and qRT-PCR were described previously (Yang et al., 2010). Primers used are listed in Supplementary Table 2.
Histological and immunohistological stainings and labeling with BrdU

Histological and immunofluorescence analyses were described earlier (Schäfer et al., 2010). For antigen retrieval, PFA-fixed paraffin sections were heated up from room temperature to 95°C and incubated for 1 h at 95°C (for SLPI, SPRR2D, and EPGN). Primary antibodies used were anti-Adph (Fitzgerald, Acton, MA, USA), anti-BrdU-POD (Roche, Rotkreuz, Switzerland), anti-PCNA, and anti-Slipi (all from Santa Cruz, CA, USA), anti-Nrf2 (Huebner et al., 2012), anti-H3K4me2 (Active Motif, Carlsbad, CA, USA), anti-Epgn, anti-NQO1 (Abcam, Cambridge, UK), anti-SPRR2 (kindly provided by Dr. Daniel Hohl, Lausanne, Switzerland), and antibodies against keratins K17, K75, K28, K82, and K33 (L. Langbein). Donkey anti-guinea pig-Cy3, goat anti-mouse-biotin (Vector Laboratories, Burlingame, CA, USA), and goat anti-rabbit-biotin (Jackson ImmunoResearch, West Grove, PA, USA) were used as secondary antibodies.

BrdU short-term labeling and detection as well as Oil Red O staining were previously described (Schäfer et al., 2012).

Preparation of epidermal whole mounts

Skin was manually peeled off from the mouse tail, cut into approximately 1-cm-long pieces, and incubated in a solution containing 2.5 U/ml dispase (Invitrogen, Carlsbad, CA, USA) in PBS for 2 h at 37°C. Epidermal sheets were separated from the dermis and fixed overnight in 4% PFA or in 95% ethanol/1% acetic acid.

Morphometrical analysis

Paraffin-embedded back skin was cut longitudinally; tail and eyelid skin were cut transversally to obtain 7-μm sections. Morphometrical analysis of the SG and meibomian gland areas was performed on Adph or hematoxylin/eosin (H&E)-stained serial transverse sections. The gland size of meibomian glands and tail SG was compared at its largest extent. Morphometrical analysis of back skin SG was performed on longitudinal sections. Only areas, where the hair follicles were cut longitudinally, were analyzed. For all analyses, the Openlab 3.1.5 software (Perkin Elmer, Waltham, MA, USA) was used.

Lipid analysis and quantification

Lipids were extracted from the back skin and analyzed as described previously. The lipids of interest, which contain free fatty acids, triacylglycerol, cholesterol, and cholesterol- and wax esters, were first separated by HPTLC and subsequently quantified by densitometry. In particular, the mentioned lipids were applied in form of thin bands (1 cm in length) automatically on 20 × 10 cm silica HPTLC plates (VWR, Germany). The bands were then separated using a solvent mixture containing n-hexane, diethyl ether, and acetic acid (70:30:1 by volume). After drying, the HPTLC plates were dipped into an aqueous solution containing 10% CuSO4 and 8% H3PO4 (w/v). Heating of the plates to 160°C for 10 min led to visualization of the separated lipids. Visualized lipid bands were quantified using a densitometric scanner (TLC scanner 3; CAMAG, Germany) in the reflectance mode at 595 nm. Quantitative results were obtained by relating the intensities of the separated lipid bands to calibration curves of corresponding standards.

Chromatin immunoprecipitation

Chromatin lysate was pre-cleared and incubated overnight with antibodies against Nrf2 (Huebner et al., 2012), Sprr2 (see above), histone H3 (Abcam, Cambridge, UK), histone H3K4me2, and normal sheep IgG (Upstate/Millipore, Billerica, MA, USA). To analyze protein-bound DNA, primers for qPCR were used (Supplementary Table 2). The percentage of the input that was bound was calculated by the delta Ct method and averaged over at least three experiments.

Animal experiments

For activation of endogenous Nrf2 in the skin, wild-type mice were topically treated twice a day for 10 consecutive days starting at P0 with 10 mM sulforaphane or 50 mM tBHQ (both from Merck, Darmstadt, Germany) in hydrophilic cream (33%) (Hänsele, Hersau, Switzerland) with DMSO (66%) (vehicle).

EGFR signaling was inhibited by intraperitoneal injection of P18 K5cre-CMVcaNrf2 and control mice with 70 μg Gefitinib (Iressa) (Santa Cruz) per gram body weight once a day for 14 consecutive days. Gefitinib was diluted in DMSO (21%) and olive oil (79%) (vehicle).

Mice were sacrificed by CO2 inhalation. Animal maintenance and experiments with animals had been approved by the local veterinary authorities of Zurich, Switzerland.

Human skin biopsies

Biopsies from affected skin of three patients with TCDD intoxication had been taken for diagnostic purposes after informed consent and with permission from the local ethics committee, Vienna, Austria, and Geneva, Switzerland. Patient 1 initial TCDD level: 144,000 pg/g blood fat, Patient 2 initial TCDD level: 26,000 pg/g blood fat, and Patient 3 initial TCDD level: 108,000 pg/g blood fat. The skin samples had been formalin-fixed and paraffin-embedded and stored in the files of the Department of Dermatology at the Medical University of Vienna since 1998 and at the University of Geneva since 2005. For the current analysis, 5-μm sections were taken and either stained with H&E or further processed for immunohistochemical staining as described above.

TCDD treatment and siRNA transfection of human foreskin keratinocytes

Primary HDFs were grown in 6-cm dishes in Keratinocyte-SFM (Gibco, Paisley, UK) supplemented with EGF and bovine pituitary extract. They were transfected at 70% confluency with 50 nM 21-mer siRNA targeting NRF2 (Sigma-Aldrich, Buchs, Switzerland) or AHR (Life Technologies, Carlsbad, CA, USA) using INTERFERin (Polyplus, Illkirch, France). After 48 h at 100% confluency, HDFs were washed with PBS and cultured for 48 h in supplement-free Keratinocyte-SFM medium with 10−8 M TCDD (Sigma-Aldrich) in toluene/DMSO (0.1% final concentration) or toluene/DMSO only.
In situ hybridization

In situ hybridization was performed with digoxigenin-labeled sense and antisense probes complementary to bp 91–839 of the murine Epgn mRNA (NM_053087.2). For this purpose, the full-length cDNA was amplified by PCR using primers that allowed the introduction of the T7 and SP6 RNA polymerase promoter regions. 400 ng of the purified PCR product was then used to synthesize digoxigenin-labeled RNA probes according to the manufacturer’s instructions (DIG RNA Labeling Kit (SP6/T7), Roche Applied Science). In situ hybridization was performed as previously described (Brackmann et al., 2013). Briefly, 14-µm-thick sections of back skin were post-fixed with 4% PFA and hybridized to the Epgn antisense and sense probes at 60°C overnight. The next day, sections were washed and blocked, followed by incubation with an anti-digoxigenin antibody conjugated to alkaline phosphatase (anti-digoxigenin-AP, Fab fragments, Roche Applied Science) overnight. Detection of hybridized probes was performed using NBT/BCIP solution (Roche Applied Science).

Replicate experiments and statistical analysis

qRT-PCR analyses were performed using cDNA of three or more mice from one litter, three or more pools of different litters, or three or more dishes of cultured cells of one experiment. qRT-PCRs using cDNA from cultured cells were repeated with independent samples. Statistical analysis was performed on samples N ≥ 3 using the non-parametric Mann–Whitney U-test for non-Gaussian distribution and the Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Error bars represent standard deviation.

Supplementary information for this article is available online: http://embomolmed.embopress.org

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Author contributions

MS performed experiments, designed the study and experiments together with SW, and wrote the manuscript together with SW; AHW, SK, HB8, ASL, HF, and WB performed experiments and analyzed the data; AG, FG, OS, ET, and JHS provided samples from MADISH patients and helped with the histological characterization; KS designed the lipid analysis experiments and helped with the data analysis; MRS provided samples from EPGN transgenic mice; AJH and DRR provided the affinity-purified Nrf2 antibody; LL provided the K28, K32, K75, K82, and K85 antibodies; SW designed the study and the experiments together with MS and wrote the manuscript together with MS. All authors made comments on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


