It is interesting to note that both Atg5 and Bcl-2 mediate many of their effects at the level of the mitochondria, and that the toxicity of the Atg5 cleavage fragment can be abrogated by Bcl-2.

Another level of complexity is introduced into this cross-talk, since the major pathway for mitochondrial clearance is via autophagy. Indeed, levels of mitochondria accumulate when autophagy is blocked, while mitochondrial load decreases when autophagy is activated. Cells show increased susceptibility to subsequent propapoptotic insults after autophagy is blocked.[30,31] Conversely, after autophagy is induced in cells (or flies), cells show increased resistance to subsequent proapoptotic insults.[30] Our data suggest that this is likely to be due to the changes in mitochondrial load resulting from perturbation of autophagy.[30] When autophagy is induced, there are fewer mitochondria after a period of $\sim$72 h (or a period of autophagy perturbation sufficient to influence steady-state levels of mitochondria), and if cells are exposed to proapoptotic insults, there is understandably less cytochrome *c* release and subsequent caspase activation. (The converse occurs when autophagy is inhibited.). Since cells can tolerate major decreases in mitochondrial load without compromise to oxidative phosphorylation, there is a significant window where decreases in mitochondrial load may have beneficial effects with regard to survival after certain toxic insults. Clearly, this mechanism would also be relevant to Atg5 fragment toxicity.

In conclusion, studies investigating molecular mechanisms of cross-talk between apoptosis and autophagy are still in their infancy. However, these provide testable hypotheses and insights into both processes. For instance, the data suggest that it may be important to examine carefully the roles of Atg5 in different types of apoptosis (e.g. in development). This is possible, as both conditional and constitutive knockout mouse models are available for this gene. Interestingly, no developmental abnormalities have been reported in such models, although these may not have been studied carefully in the initial analyses.[22] Further insights into the mechanism by which Atg5 enhances apoptosis will also be revealed. Does the Atg5 fragment bind Bcl-2, in addition to Bcl-xl, and which domains of these proteins interact with Atg5? Does Atg5 fragment binding prevent Bcl-2 and Bcl-xl from sequestering pro-death Bcl-2 family members? Deeper insights into these and related interplays between autophagy and apoptosis are likely to have important implications for our understanding of both process in development, normal physiology and disease.

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Roles and mechanisms of action of the Nrf2 transcription factor in skin morphogenesis, wound repair and skin cancer

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The Nrf2 transcription factor plays a key role in the cellular defense against oxidative and xenobiotic stresses through its capability to induce the expression of genes, which encode detoxifying enzymes and antioxidant proteins. Most interest-

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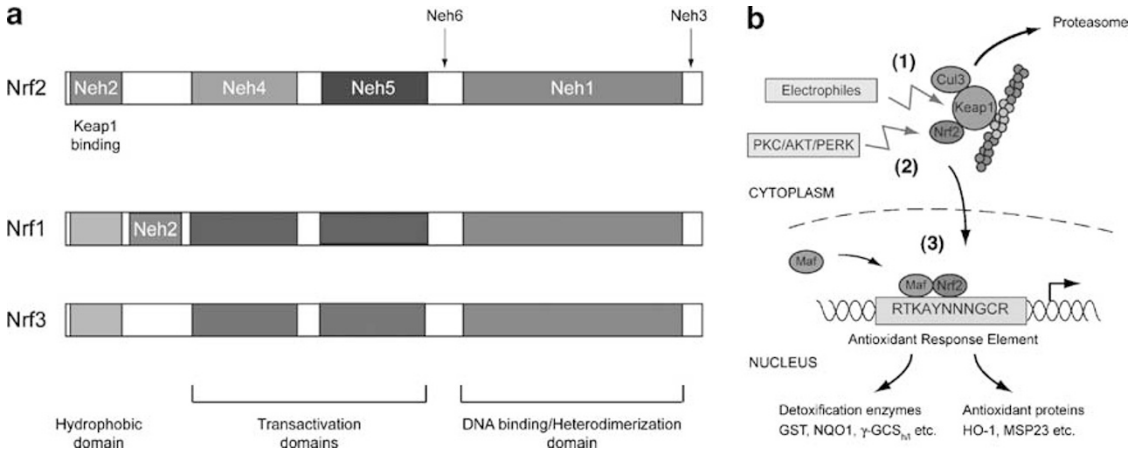

Figure 1 (**a**) Domain composition of Nrf transcription factors. Neh1 comprises the CNC/bZip domain and the nuclear localization signal and is conserved in all three family members. It is responsible for protein–protein interaction and DNA binding. Further, all Nrf transcription factors have two independent transactivation domains, which are conserved to a certain extent. The Neh2 domain is present in Nrf1 and Nrf2, and it is responsible for the cytoplasmic retention of Nrf2 by Keap1. (**b**) Schematic representation of the Nrf2/Keap1 system. In response to electrophiles, which directly react with Keap1 (1), or upon phosphorylation of Nrf2 by PKC, Akt kinase or PERK (2), Nrf2 is stabilized, liberated from Keap1 and translocates to the nucleus (3). Upon heterodimerization with a small Maf protein, Nrf2 binds to AREs in the promoters of its target genes, which encode – among others – ROS-detoxifying enzymes and antioxidant proteins

ingly, recent studies provide evidence for an important function of Nrf2 in the protection against various severe diseases, including neurodegenerative disorders, chronic inflammatory diseases and cancer. However, a role of Nrf2 in skin biology and pathology has only recently emerged. Here, we report on the roles and mechanisms of action of Nrf2 in skin morphogenesis, wound repair and skin cancer.

The skin is frequently exposed to ultraviolet (UV) irradiation or toxic chemicals, which induce the formation of reactive oxygen species (ROS). These aggressive molecules damage cellular macromolecules, resulting in premature aging, severe tissue destruction or even carcinogenesis. Large amounts of ROS are also generated by inflammatory cells in skin wounds as a defense against bacterial infection. Although this activity is beneficial, ROS can damage the inflammatory cells themselves and other cells at the wound site. Therefore, cells had to develop strategies to detoxify ROS, such as production of low molecular weight antioxidants and expression of ROS-detoxifying enzymes. Interestingly, several genes encoding such enzymes are under the control of the NF-E2-related factor 2 (Nrf2). Nrf2 is a member of the 'cap 'n' collar' family of transcription factors, which also includes the related Nrf1 and Nrf3 proteins (Figure 1a), as well as p45 NF-E2, Bach1 and Bach2.[1] Upon heterodimerization with small Maf proteins or other leucine zipper proteins, Nrf2 binds to cis-acting elements in the promoters of its target genes, called antioxidant response element (ARE) or electrophile response element. At least the binding of Nrf1 and Nrf2 activates the expression of these genes, which encode, for example, NADPH:quinone oxidoreductase (NQO1), several glutathione *S*-transferases (GST), γ-glutamyl-cysteine synthetase heavy subunit (γ-GCS$_h$) and light subunit (γ-GCS$_l$), and heme oxygenase 1 (HO-1) (Figure 1b; reviewed by Nguyen *et al.*[2]).

Nrf2 is normally retained in the cytoplasm via interaction with Keap1, an actin-binding protein, which also mediates its degradation via the ubiquitin–proteasome pathway.[3,4] Activation of Nrf2 can be achieved by electrophilic chemicals, which react with Keap1 through Michael addition. This results in stabilization of Nrf2 and its accumulation in the nucleus, where it activates its target genes (Figure 1b; reviewed by Itoh *et al.*[5] and Nguyen *et al.*[2]). In addition, there is evidence that pro-oxidants can also activate Nrf2, possibly via oxidation of Keap1 and/or stimulation of Nrf2 phosphorylation by different kinases, including protein kinase C (PKC), Akt and PKR-like endoplasmic reticulum kinase (PERK) (Figure 1b; reviewed by Nguyen *et al.*[2]).

The important role of Nrf2 in the cellular stress response is reflected by the phenotype of Nrf2 knockout mice. Upon aging, these mice develop a severe autoimmune disease resembling systemic lupus erythematosus.[6] Furthermore, even young Nrf2-deficient animals are highly susceptible to electrophilic and oxidative stress exerted by various chemicals, and the loss of Nrf2 enhances their susceptibility to several pathologies, including cancer (reviewed by Itoh *et al.*[5]). In addition to Nrf2, a role of Nrf1 in the regulation of ROS detoxification has also been reported. Mice deficient in this transcription factor die at midgestation as a result of anemia due to presumed developmental arrest in fetal liver erythropoiesis.[7] In a chimeric analysis, Nrf1-deficient embryonic stem cells contributed to fetal, but not adult liver cells. In late-gestation chimeric fetuses, strong apoptotis of liver cells was observed, most likely as a result of increased oxidative stress.[8] Further, mice lacking Nrf1 in the adult liver revealed enhanced levels of ROS in hepatocytes, and they developed severe steatosis and spontaneous liver cancer.[9] The biological function of Nrf3 has as yet not been determined and mice deficient in this transcription factor develop normally and do not reveal any obvious abnormalities.[10]

Interestingly, a role of Nrf transcription factors in homeostasis, repair and disease of the skin has only recently been identified, and these results are summarized in this article.

Expression of Nrf2 in Normal, Wounded and Diseased Skin

A first indication for a role of Nrf2 in the skin was the identification of the *Nrf2* gene as a target of keratinocyte growth factor (KGF) in keratinocytes.[11] Since KGF is a cytoprotective growth factor for epithelial cells, which is highly expressed in skin wounds, this finding suggested that Nrf2 may also be expressed and potentially upregulated in keratinocytes after injury. Indeed, in full-thickness excisional mouse wounds, a strong increase in Nrf2 expression was observed compared to normal skin. Nrf2 mRNA was predominantly found in keratinocytes of the wound epidermis and in macrophages. By contrast, expression of Nrf3 was slightly downregulated in wounded skin, whereas the expression of Nrf1 was not affected by wounding.[11] Using microarray analysis of RNA from keratinocytes of mouse incisional skin wounds, which had been isolated by laser capture micro-dissection, the upregulation of Nrf2 expression in the wound epidermis compared to normal epidermis was confirmed.[12]

Nrf2 Regulates Inflammation but not Reepithelialization of Skin Wounds

To determine a possible function of Nrf2 in the wound-healing process, we generated full-thickness excisional wounds in Nrf2 knockout mice but could not observe any obvious macroscopic or histological abnormalities. A more detailed molecular and cellular analysis revealed reduced expression of several cytoprotective Nrf2 target genes and prolonged presence of macrophages at the wound site. As a result, expression of pro-inflammatory cytokines was enhanced at later stages of the repair process. However, wound re-epithelialization was not affected, in spite of the high levels of Nrf2 produced by wound keratinocytes. Interestingly, a striking increase in Nrf3 expression was seen in normal and wounded skin of Nrf2 knockout mice compared to wild-type littermates, and the hyperproliferative wound epidermis was identified as the major site of Nrf3 expression.[11] This finding suggested that the loss of Nrf2 is compensated by upregulation of Nrf3 in keratinocytes. To test this hypothesis, we generated transgenic mice expressing a dominant-negative Nrf2 mutant (dnNrf2) in basal keratinocytes of the epidermis under the control of the keratin 14 promoter (designated K14-dnNrf2 mice).[13] DnNrf2 lacks both transactivation domains and the Keap1 binding domain, but includes the DNA binding domain. Therefore, it is expected to continuously bind to AREs, thereby competing with endogenous Nrf1, Nrf2 and Nrf3. The functionality and specificity of the mutant was verified *in vitro* and *in vivo*. Surprisingly, skin morphogenesis and wound healing were not affected in the transgenic mice, and even the re-epithelialization process occurred normally. This finding suggested that the Nrf2 present in wound keratinocytes is not activated. To address this question we generated full-thickness excisional wounds in transgenic ARE reporter mice. The latter harbor a reporter construct in their genome that includes a 51 bp ARE containing fragment of the rat NQO1 promoter upstream of an initiator element containing minimal promoter, followed by the human placental alkaline phosphatase (hPAP) cDNA.[14] Therefore, they allow to monitor ARE activation *in vivo* by staining for hPAP activity. Consistent with the normal wound re-epithelialization in K14-dnNrf2 mice, there was no activation of the reporter in the wound epidermis at any stage of healing. However, a strong reporter activity was seen in cells of the granulation tissue, which predominantly represent inflammatory cells.[13]

Taken together, these findings demonstrate that Nrf-mediated gene expression in keratinocytes is dispensable for wound repair, at least under normal laboratory conditions. It remains to be determined if Nrf2 is activated under more harsh conditions, such as in infected wounds or in chronic human ulcers and if Nrfs are crucial for the healing of these types of wounds.

Nrf2 in the Epidermis is Crucial for Skin Tumor Prevention

We subsequently used the K14-dnNrf2 transgenic mice to determine if Nrf-mediated gene expression in keratinocytes is important in a situation with abnormal and continuous hyperproliferation of the epidermis. For this purpose we used the well-characterized model of two-stage chemical carcinogenesis in mouse skin. In this approach, mice are treated once topically with the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA), which causes mutations in the DNA, including a specific oncogenic mutation in the *ha-ras* proto-oncogene. This is followed by a weekly treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), a growth-promoting agent. The treatment starts by inducing papillomas, and over time some of these will progress to form squamous cell carcinomas. Remarkably, K14-dnNrf2 mice started to show papillomas much earlier compared to age- and sex-matched wild-type controls. Furthermore, the number of papillomas per animal was significantly higher in transgenic compared to wild-type animals. At 20–40 weeks after the first treatment, malignant conversion was observed in a subset of papillomas from mice of both genotypes, but the frequency of conversion was not affected by the dnNrf2 mutant. Therefore, the loss of Nrf-mediated gene expression enhanced the rate of tumorigenesis, but did not affect the phenotype and malignancy of the tumors.[13]

Subsequently, ARE reporter mice were also subjected to the same skin carcinogenesis protocol, but reporter activation was not detectable at any time point.[13] This finding strongly suggests that the basal but not the inducible expression of Nrf target genes is responsible for the tumor-preventive effect of Nrf transcription factors in the skin. Since Nrf2 also regulates the basal expression of several target genes in keratinocytes,[11] we propose that the long-term reduction in the basal levels of ROS-detoxifying enzymes results in a continuous increase in intracellular ROS and accumulation of oxidative damage. Consistent with this hypothesis, expression of several enzymes involved in ROS detoxification was reduced in papillomas and adjacent non-tumorigenic skin of K14-dnNrf2 transgenic animals. The latter include γ-GCS, the rate-limiting enzyme in glutathione biosynthesis, as well as NQO1, which prevents accumulation of ROS through its ability to circumvent redox cycling of quinones. The reduced expression of these enzymes is likely to enhance the oxidative stress in the presence of DMBA and TPA, and this hypothesis is

supported by the elevated levels of oxidized proteins that were found in papillomas as well as in the adjacent non-tumorigenic, DMBA-/TPA-treated skin of our K14-dnNrf2 transgenic animals. In addition, the expression of genes, which encode the DMBA-detoxifying enzymes NQO1 and GST-π, was also reduced in papillomas as well as in the long-term DMBA-/TPA-treated non-tumorigenic skin of transgenic mice.[13]

The reduced detoxification of DMBA and ROS is likely to enhance the rate of mutations in keratinocytes, and ROS accumulation during the promotion phase will speed up tumorigenesis.

Since the dominant-negative Nrf2 mutant blocks ARE binding of all Nrf transcription factors, the contribution of the individual members of the Nrf family to the tumor-preventive effect could not be determined in this study. However, when ARE-hPAP reporter mice were mated with Nrf2 knockout mice, activation of the reporter by the electrophilic chemical tert-butylhydroquinone (tBHQ) was completely abrogated in keratinocytes and fibroblasts.[13] This finding demonstrates that Nrf2 comprises the major ARE-binding activity upon activation by tBHQ in these cell types. Nevertheless, it is still possible that in addition to Nrf2, the basal activities of the other family members also contribute to this effect or that other stimuli are required to activate these factors.

An answer to this question was provided in a recent study by Xu *et al.*,[15] who subjected Nrf2 knockout mice to the DMBA/TPA skin carcinogenesis protocol. Similar to the findings obtained with the K14-dnNrf2 transgenic mice, Nrf2 knockout mice also revealed a higher tumor incidence and multiplicity after DMBA/TPA treatment. Therefore, the results of both studies demonstrate that Nrf2 is important for prevention of epidermal cancer and that it exerts this effect in a cell-autonomous manner and not indirectly via stromal cells.[13,15]

Interestingly, reduced expression or even complete loss of Nrf2 and its target HO-1 were found in the DMBA-/TPA-induced skin tumors of wild-type mice, suggesting that skin papilloma formation in mice is associated with downregulation of endogenous Nrf2 and its target genes.[15] On the other hand, upregulation of Nrf2 was observed in the rather malignant squamous cell carcinomas of human skin.[12] Therefore, it will be interesting to determine if malignant progression of skin papillomas to squamous cell carcinomas, which occurs at later stages in the DMBA/TPA mouse carcinogenesis model, is associated with upregulation of Nrf2. This could be a mechanism to enhance malignant progression and resistance to chemotherapy. Consistent with this hypothesis, inactivating mutations in the *Keap1* gene have recently been detected in non-small-cell lung cancer, resulting in hyper-activation of Nrf2. As a consequence, expression of antioxidants, detoxification enzymes and drug transporters was enhanced, resulting in higher malignancy and chemoresistance.[16]

Constitutive Activation of Nrf2 in Keratinocytes is Deleterious

The results obtained in the above-described studies suggest that activation of Nrf2 in normal cells is beneficial, whereas hyperactivation in tumor cells may be deleterious. Therefore, it is an interesting question to determine the consequences of Nrf2 activation *in vivo*. Since Nrf2 activity is inhibited by Keap1, Wakabayashi *et al.*[17] deleted this gene in mice. Surprisingly, Keap1 knockout mice die within the first 3 weeks after birth due to hyperkeratosis in the esophagus and stomach, resulting in nutrient obstruction and stomach ulceration. These mice also revealed severe scaling and hyperthickening of the cornified layer of the epidermis. Increased expression of the differentiation markers keratin 1, keratin 6 and loricrin was detected in keratinocytes of the esophagus and forestomach of the Keap1 knockout mice. Since the genes that encode these proteins of stratified epithelia contain AREs, they may be directly regulated by the hyperactive Nrf2. This hypothesis was further supported by the fact that breeding of Keap1 null mice with Nrf2 knockout animals or with mice lacking the Nrf2 dimerization partners MafG and MafF rescued the defect in the skin and esophagus.[17,18] Therefore, inhibition of Nrf2 action is obviously the predominant function of Keap1. Furthermore, this finding suggests that the differentiation abnormalities result from binding of active Nrf2 to the promoters of differentiation-specific genes in keratinocytes and that constitutive activation of Nrf2 in the epidermis is detrimental due to abnormal enhancement of keratinocyte differentiation.

Short-Term Activation of Nrf2 in the Skin has a Cancer-Preventive Effect

Although constitutive activation of Nrf2 in keratinocytes is obviously a disadvantage, short-term activation or continuous weak activation of Nrf2 may be beneficial. Indeed, topical application of broccoli sprout extract, which includes the electrophilic Nrf2 activator sulforaphane, protected mice against UVB- or chemically induced skin carcinogenesis.[19,20] This effect appears to be at least in part mediated through Nrf2, since a 14-day pre-treatment of the skin with sulforaphane decreased the incidence of DMBA-/TPA-induced skin tumors in wild-type mice but not in Nrf2 knockout animals,[15] and the cancer-preventive effect in wild-type mice was associated with upregulation of Nrf2 protein.[15] A similar protective effect for the skin was observed with triterpenoid electrophiles (avicins). In this study avicins reduced the deleterious effects of long-term UVB treatment, including epidermal hyperplasia, mutations in the *p53* gene and formation of 8-hydroxy-2′-deoxyguanosine. In addition, apoptosis of keratinocytes and expression of ROS-detoxifying enzymes was enhanced.[21] Therefore, avicins were identified as promising substances for skin protection under stress conditions.

Electrophilic Chemicals are Potent Activators of Nrf2 in Keratinocytes

The potent cancer-preventive effect of sulforaphane underscores the importance to determine the mechanisms of Nrf2 activation in keratinocytes and to identify potent and specific substances, which activate Nrf2 in these cells. One possibility to achieve this goal is through inhibition of Keap1 as demonstrated previously for keratinocytes, where siRNA-mediated Keap1 knockdown induced the expression of cytoprotective genes.[22] Alternatively, direct activation of Nrf2 by certain chemicals could be a suitable approach. Electrophilic chemicals have been described as activators of

Nrf2 in several cell types (for review see Itoh *et al.*[5]). Using reporter assays with cultured keratinocytes from ARE-hPAP reporter mice as well as analysis of Nrf2 target gene expression and of nuclear translocation of Nrf2, we confirmed that the electrophiles tBHQ and sulforaphane activate Nrf2 in this cell type.[23] Electrophiles can directly react with Keap1 via Michael addition[2] thereby inhibiting the Keap1-mediated proteasomal degradation of Nrf2 and accumulation of stabilized Nrf2 in the nucleus.[24,25] However, they also induce ROS formation through redox cycling.[23] This activity, however, is obviously not responsible for the Nrf2 activation, since a short (2-h) treatment with tBHQ enhanced the expression of known Nrf2 target genes but not the levels of intracellular ROS. Furthermore, ARE-mediated gene expression in keratinocytes by tBHQ was not abrogated when the cells were pre-treated with the antioxidant *N*-acetylcysteine, although this treatment abolished the tBHQ-mediated increase in intracellular ROS.[23]

Whereas the activation of Nrf2 by electrophiles is generally accepted, the role of ROS in Nrf2 activation is still under debate. For example, ARE activation was reported in response to hyperoxia or to H_2O_2 or glucose oxidase.[26,27] By contrast, we did not observe activation of Nrf2 by different concentrations of H_2O_2 or by glucose oxidase, which catalyzes the continuous production of ROS.[23] The discrepancy between the published results and our data may reflect cell-type specific differences in Nrf2 activation. In support to this hypothesis, we found that primary murine fibroblasts and macrophages from ARE reporter mice already express the reporter gene under normal culture conditions in the absence of electrophiles or exogenous ROS. Surprisingly, however, activation of Nrf2 in the human immortalized HaCaT keratinocyte cell line was observed in response to inorganic arsenic, and this effect was shown to involve H_2O_2. Furthermore, H_2O_2 alone induced nuclear accumulation of Nrf2.[28] However, it is not known if Nrf2 target genes are indeed activated in response to H_2O_2 in HaCaT keratinocytes.

Interestingly, UVB irradiation (10–40 mJ/cm^2) also failed to activate the ARE-hPAP reporter in mouse keratinocytes, although elevated levels of ROS were observed under these conditions.[23] However, low doses of UVB (5–7.5 mJ/cm^2) induced nuclear translocation of Nrf2 as well as Nrf2-mediated gene expression in a mouse keratinocyte cell line established from skin tumors. In the same cell line, high doses of UVB resulted in nuclear exclusion of Nrf2.[29] It will be interesting to determine if UVB also has a dual effect on Nrf2 activity in non-tumorigenic keratinocytes. A cell-type specificity in Nrf2 activation was observed for UVA, which induced nuclear translocation and accumulation of Nrf2 in cultured fibroblasts,[30] but failed to activate Nrf2 in mouse keratinocytes.[23]

Activation of Nrf2 in keratinocytes by electrophiles, but not by ROS or UVB, was also demonstrated *in vivo* using ARE reporter mice.[23] In these experiments, only tBHQ but not UVB activated the ARE-hPAP reporter in keratinocytes of hyperproliferative mouse skin. Consistent with this finding, we only found ARE activation in inflammatory cells of the granulation tissue of skin wounds, but not in keratinocytes (auf dem Keller *et al.*[13]; see above), although these cells are also exposed to large amounts of inflammatory cell-derived ROS in the wound environment. This suggests that keratinocytes have developed mechanisms to prevent ROS-induced Nrf2 activation. This could be an important prerequisite for their survival, since continuous activation of Nrf2 in keratinocytes is deleterious as demonstrated by the lethal phenotype of Keap1 knockout mice.[17] Although the mechanisms underlying such differences in Nrf2 activation between keratinocytes and other cell types remain to be determined, most of the published data indicate that electrophiles are the most potent activators of Nrf2 in keratinocytes. This result provides an important basis for the identification of novel Nrf2 activators in this cell type, which can be used for skin protection under stress conditions.

Conclusions

The results described in this article demonstrate important functions of Nrf2 in the skin:

1. Endogenous Nrf2 is not required for skin morphogenesis, homeostasis and wound re-epithelialization, but it regulates inflammation in wounded skin. Most importantly, it protects from chemically induced skin carcinogenesis.
2. Constitutive activation of Nrf2 in the epidermis is deleterious due to induction of keratinocyte differentiation. By contrast, transient activation of Nrf2 in normal skin protected from UVB- and toxin-induced skin cancer. Thus, controlled and transient activation of Nrf2 in healthy skin may present a novel strategy for skin protection under stress conditions.

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