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Amniotic Fluid Activates the Nrf2/Keap1 Pathway to Repair an Epidermal Barrier Defect In Utero

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SUMMARY

The loss of loricrin, a major component of the cornified envelope, results in a delay of epidermal barrier formation. Therefore, the living layers of the epidermis are aberrantly exposed to late-stage amniotic fluid, which may serve as the signal to upregulate genes that functionally compensate for the loss of loricrin. Consistent with this hypothesis, metabolomic studies revealed marked changes in amniotic fluid between E14.5 and E16.5 days postcoitum. In addition, we discovered that the Nrf2/Keap1 pathway detects these compositional changes and directly upregulates the expression of genes involved in the compensatory response, thus ensuring postnatal survival. In support of this finding, we demonstrate that genetically blocking the Nrf2 pathway abolishes the compensatory response and that preemptively activating Nrf2 pharmacologically rescues the delay in barrier formation in utero. Our findings reveal that the functions of Nrf2 and the composition of amniotic fluid have coevolved to ensure the formation of a functional barrier.

INTRODUCTION

The epidermis is the interface between an organism and the outside environment, protecting the body from dehydration and infection. The acquisition of an epidermal barrier occurs late in mammalian gestation and coincides with the terminal differentiation of keratinocytes (Hardman et al., 1998). This specialized process of differentiation results in the formation of a layer of flattened, enucleated cells that replace their plasma membranes with an insoluble protein and lipid matrix termed the cornified envelope (CE) (Rice and Green, 1977). Defects in the processes that generate CEs lead to severe complications in humans and postnatal lethality in genetically modified mice (Roop, 1995).

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ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the loricrin knockout microarray data reported in this paper is GSE41742.

SUPPLEMENTAL INFORMATION

Supplemental information includes two figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.11.002.

The main protein component of the CE is loricrin, a glycine-/serine-rich structural protein that comprises >70% of the protein mass fraction of the CE (Mehrel et al., 1990; Hohl et al., 1991). Several other proteins are also crosslinked into the CE, including involucrin (Simon and Green, 1984), members of the small proline-rich protein family (Sprrs) (Kartasova et al., 1988; Hohl et al., 1995), and members of the late cornified envelope protein family (Lces) (Marshall et al., 2001).

Because of the abundance of loricrin in the CE, we assumed that loss of loricrin would result in a failure to form a functional barrier. Surprisingly, loricrin knockout (LKO) mice formed a functional barrier by birth and survived; however, the normal acquisition of barrier at E16.5 was delayed by 24 hr (Koch et al., 2000). This finding suggested that the loss of loricrin was compensated for in utero. Consistent with this hypothesis, we found that the Sprr2 family members Sprr2d and Sprr2h were transcriptionally upregulated in the epidermis of LKO mice (Koch et al., 2000).

In our search for a potential mechanism that could sense a barrier defect in utero, we became intrigued by the ubiquitously expressed transcription factor NF-E2-related factor 2 (Nrf2) and its negative regulator Kelch-like ECH-associated protein 1 (Keap1) (Zhang, 2006). Nrf2 is a master regulator of the cellular antioxidant response by transactivating the expression of various cytoprotective genes via binding to an enhancer sequence known as the antioxidant response element (ARE) (Moi et al., 1994). During homeostasis, Keap1 constitutively facilitates the ubiquitination and subsequent proteolytic degradation of Nrf2 (Itoh et al., 1999). However, during stress, such as exposure to UV radiation and/or xenobiotic stress, cells generate reactive oxygen species (ROS) and electrophiles (Nguyen et al., 2004). These electrophiles form adducts on highly reactive cysteine residues on the surface of Keap1, which results in a retardation of the Nrf2 binding pocket. As a consequence, Nrf2 accumulates and translocates to the nucleus, where it binds to AREs that reside in the regulatory regions of Nrf2-responsive genes. Stratified epithelia have evolved to utilize the stress-induced activation of Nrf2 in unique ways. This was first appreciated in the Keap1 KO, which exhibits constitutive Nrf2 stabilization and activation of Nrf2 target genes. Unexpectedly, these mice developed a progressive hyperkeratosis in the epidermis along with a cornification of the esophagus and forestomach, the latter leading to starvation and, ultimately, death. Upon further investigation, functional AREs were found in the promoters of loricrin and the stress-induced intermediate filament, keratin 6a (Wakabayashi et al., 2003). Thus, this study revealed that in addition to the classically studied role of phase II detoxification enzyme activation, Nrf2 could also activate epithelial-specific genes in a tissue-dependent manner upon stress.

During development, the developing epidermis is directly exposed to amniotic fluid. Therefore, before the barrier is formed, amniotic fluid must resemble intracellular fluid to maintain osmotic equilibrium. However, once the barrier is formed, the composition of amniotic fluid can change with no effect on the living layers of the epidermis. Because barrier formation in LKO mice is delayed by 24 hr, electrophiles present specifically in late-stage amniotic fluid—normally blocked by the epidermal barrier—may diffuse into LKO keratinocytes, react with Keap1, and stabilize Nrf2. Thus, aberrant exposure to amniotic fluid could induce the compensatory response via the Nrf2/Keap1 pathway in LKO mice. Here we present data that strongly support this unexpected mechanism.

RESULTS

E16.5 Amniotic Fluid Stabilizes Nrf2 In Vitro and In Vivo, Resulting in the Activation of Nrf2 Target Genes

To determine if changes in the composition of amniotic fluid coincided with normal epidermal barrier formation, we performed metabolomic profiling on E14.5 and E16.5 amniotic fluid. Collectively, we found >200 distinct metabolites that were differentially present between E14.5 and E16.5 days postcoitum amniotic fluid (data not shown). Twelve of these metabolites were found exclusively in E16.5 amniotic fluid (Table S1 available online).

To determine whether compounds present in E16.5 amniotic fluid could activate the Nrf2/Keap1 pathway, we utilized a transgenic mouse line that can detect and report Nrf2 activity by colorimetric assay. This transgenic mouse expresses human placental alkaline phosphatase (hPAP) via a minimal promoter and the core ARE enhancer taken from the rat NADP(H) dehydrogenase quinone 1 (Nqo1) promoter, a known and well-studied downstream target of Nrf2 (AREhPAP) (Johnson et al., 2002). We isolated and exposed primary AREhPAP^{+} keratinocytes to 1.2 mM Ca^{2+}, E14.5 amniotic fluid, E15.5 amniotic fluid, E16.5 amniotic fluid, DMSO, or 30 μM tert-butylhydroquinone (tBHQ), a known Nrf2 activator. Exposure of AREhPAP^{+} keratinocytes to E15.5 amniotic fluid resulted in low levels of hPAP activity, whereas exposure to E16.5 amniotic fluid resulted in a high percentage of keratinocytes that stained for hPAP activity, similar to tBHQ exposure (Figure 1A). In addition to activation of the hPAP reporter, the keratinocytes changed morphologically when exposed to E16.5 amniotic fluid, resembling the early stages of differentiation. To determine if E16.5 amniotic fluid initiated terminal differentiation, we examined the levels of K14, a marker of undifferentiated keratinocytes, and K1, a marker of terminal differentiation, by western blot analysis (Figure S1A). Consistent with the morphological changes, treatment with E16.5 amniotic fluid resulted in a decrease in expression of K14 and an induction of K1.

To confirm that the activity seen in AREhPAP^{+} keratinocytes was due to the stabilization and nuclear accumulation of Nrf2, we performed western blot analysis on nuclear extracts from primary keratinocytes exposed to amniotic fluid. Using an affinity-purified rabbit anti-mouse Nrf2 antibody (Figure S1B), we confirmed the nuclear accumulation of Nrf2 upon exposure to E16.5 amniotic fluid (Figure 1B). Consistent with previous studies documenting Nrf2 as being primarily regulated posttranscriptionally, only a low level of Nrf2 induction was observed by real-time PCR analysis following exposure to tBHQ and E16.5 amniotic fluid (Figure S1C). However, we saw a decrease in Keap1 expression following exposure to E16.5 amniotic fluid, which would further contribute to stabilization of Nrf2 (Figure S1C).

To determine whether Nrf2 was stabilized and active in vivo in E16.5 LKO epidermis, we performed RT-PCR analysis on RNA taken from skin of E16.5 LKO and wild-type (WT) mice. Several known downstream targets of Nrf2 were found to be upregulated in LKO mice, including Nqo1, glutathione S-transferase ρ (Gstρ), peroxiredoxin 1 (Prdx1), and glutamate cysteine ligase catalytic subunit (Gclc) (Figure 1C). Next, we performed immunohistochemistry using the affinity-purified Nrf2 antibody and detected ectopic nuclear accumulation of Nrf2 in the suprabasal layers of LKO epidermis, the cell layers most likely to interface with amniotic fluid (Figure 1D). Consistent with the hypothesis that amniotic fluid must resemble intercellular fluid before an epidermal barrier is formed, nuclear Nrf2 staining was not observed in either WT or LKO epidermis at E14.5 or E15.5 (Figure S1D).

If aberrant exposure to late-stage amniotic fluid is the driving force behind the compensatory response seen in the LKO, then other mouse models with in utero barrier defects should also exhibit activation of Nrf2. Therefore, we analyzed the flaky tail mouse (ft/ft), which lacks the major barrier component, filaggrin (Fallon et al., 2009), and was also shown to have a barrier defect in utero (Okano et al., 2012). Consistent with our hypothesis, a pronounced nuclear accumulation of Nrf2 was also observed in the suprabasal layers of E17.5 ft/ft epidermis (Figure 1E).

Nrf2 Is Required for the Compensatory Response in the LKO

To determine if Nrf2 activity was critical for the compensatory response in the LKO, we generated a transgenic mouse line expressing the DNA binding domain of Nrf2 under the control of the loricrin promoter (ΔNrf2) (Figure 2A). The ability of this dominant-negative Nrf2 to block endogenous Nrf2 activity in the epidermis of transgenic mice was previously demonstrated (auf dem Keller et al., 2006). We generated two different ΔNrf2 founder lines that showed robust nuclear Nrf2 staining in the granular layer, consistent with the expression of loricrin (Figure 2B). Western blot analysis of differentiated primary $\Delta Nrf2^{+}$ keratinocytes showed a dramatic overexpression of ΔNrf2 compared to endogenous levels of Nrf2 (Figure 2C). $\Delta Nrf2^{+}$ mice were born phenotypically normal (Figures 2D, 2E, and S2), confirming that Nrf2 is dispensable for normal epidermal development and homeostasis (Chan et al., 1996). To determine if Nrf2 orchestrates the compensatory response in the LKO, we crossed ΔNrf2 mice into the LKO background. ΔNrf2/LKO mice were born with a cellophane-like skin that began to crack within a few hours after birth, resulting in death by apparent desiccation within 24 hr (Figure 2D). To confirm the presence of a barrier defect in ΔNrf2/LKO mice, we examined E19.5 ΔNrf2/LKO mice histologically and by dye exclusion assay. Histological analysis revealed a failure to form a normal stratum corneum (Figure 2E, upper panels), and dye exclusion assays confirmed that the LKO/ΔNrf2 stratum corneum was unable to form a competent barrier (Figure 2E, lower panels). The defect in ΔNrf2/LKO mice appears to be confined to the uppermost layers of the epidermis because the staining patterns for K14, K10, and involucrin were comparable to age-matched WT mice (Figure S2).

Previous studies have suggested that the isothiocyanate (*R,S*)-sulforaphane (SF), a potent inducer of known Nrf2 target genes (Zhang et al., 1992), can cross the placental blood barrier and activate Nrf2 in the epidermis of developing mouse embryos (Kerns et al., 2007). Therefore, we wanted to determine if we could preemptively activate the Nrf2/Keap1 pathway with SF and rescue the delay in barrier formation in LKO embryos. Following intraperitoneal (i.p.) injection of SF in timed pregnant mice, we confirmed that SF crosses the placental blood barrier, resulting in a nuclear accumulation of Nrf2 in the developing epidermis (Figure 2F). Next, we timed pregnancies of $Lor^{+/-}$ females crossed to LKO males, injected the mothers with SF at E14.5 and E15.5, and isolated the embryos at E16.5. Dye penetration assays revealed a complete lack of barrier in E16.5 LKO embryos treated with vehicle alone, whereas E16.5 LKO embryos treated with SF were similar to their $Lor^{+/-}$ littermates (Figure 2G).

Sprr2d and Sprr2h Are Direct Downstream Targets of Nrf2

Consistent with our previous data linking the LKO compensatory response to Sprr2d and Sprr2h upregulation (Koch et al., 2000), microarray analysis of newborn $Lor^{+/-}$ and LKO mice (GEO accession number GSE41742) revealed that members of the Sprr2 gene family were the most significantly upregulated CE components in LKO mice (Table S2). Therefore, we analyzed the Sprr2d and Sprr2h genes and found putative AREs in each of their proximal promoters (Figure 3A). Both promoters also contain an ARE-like AP1 site that cannot bind Nrf2 because of slight sequence differences between the two promoter elements. To confirm

the ability of Nrf2 to bind each of the putative AREs, we performed chromatin immunoprecipitation (ChIP) using our affinity-purified Nrf2 antibody and lysates from differentiated primary keratinocytes that had been treated with tBHQ. We compared the binding affinity of Nrf2 to the Sprr2d and Sprr2h AREs with binding to the ARE found in the mouse Nqo1 promoter. We found that Nrf2 occupies the Sprr2d and Sprr2h AREs with similar affinity as the Nqo1 ARE when keratinocytes were exposed to tBHQ. This contrasted with the Sprr2d and Sprr2h AP1 sites, which were not occupied by Nrf2 (Figure 3B).

To confirm that each of the AREs were functional, we generated reporter constructs corresponding to the ARE sequences found in Sprr2d and Sprr2h, as well as constructs with these core ARE sequences mutated (ΔARE). Primary keratinocytes were transfected with each of these constructs and treated with tBHQ to stabilize endogenous Nrf2. Compared to the ΔARE constructs, both of the Sprr2d and Sprr2h ARE constructs exhibited a significant increase in luciferase activity (Figure 3C), suggesting that each ARE binding site was functional.

Our previous analysis suggested that members of the Sprr2 family were major constituents of LKO CEs (Jarnik et al., 2002). To confirm the role of Sprr2d and Sprr2h in the compensatory response, we performed real-time PCR on RNA from the skin of newborn WT, LKO, and ΔNrf2/LKO mice. In addition, we examined Sprr2d and Sprr2h expression in the ft/ft mice because these mice also showed a stabilization and nuclear accumulation of Nrf2 in utero. Both LKO mice and ft/ft mice showed a significant increase in both Sprr2d and Sprr2h transcripts. In contrast, transcripts of both Sprr2d and Sprr2h were decreased to basal levels in ΔNrf2/LKO mice, which is consistent with these genes playing an important role in the compensatory response (Figure 3D).

To determine if the increase in Sprr2d and Sprr2h expression correlated with increased Sprr2 protein levels, we performed immunohistochemistry using a Sprr2-specific antibody (Hohl et al., 1995). Consistent with the transcriptional data, Sprr2 protein levels were dramatically increased in both the LKO and ft/ft mice. Additionally, the ΔNrf2/LKO mice showed levels of Sprr2 that were comparable to WT mice, further suggesting that Nrf2 is required for the compensatory induction of Sprr2d and Sprr2h in the LKO (Figure 3D, lower panels).

Late-Stage Amniotic Fluid Induces Sprr2d and Sprr2h Expression via Nrf2

Because E16.5 amniotic fluid can induce Nrf2 activation in vitro, and Nrf2 can bind to functional AREs in the promoters of Sprr2d and Sprr2h to drive their expression, we wanted to determine if keratinocytes exposed to amniotic fluid resulted in Nrf2-dependent expression of Sprr2d and Sprr2h. Therefore, we exposed primary keratinocytes from WT or $\Delta Nrf2^{+}$ mice to E14.5 and E16.5 amniotic fluid in the presence or absence of calcium and measured Sprr2d and Sprr2h mRNA levels by real-time PCR. The transcript levels of both Sprr2d and Sprr2h dramatically increased upon exposure to E16.5 amniotic fluid. However, when we exposed $\Delta Nrf2^{+}$ primary keratinocytes to E16.5 amniotic fluid, upregulation of Sprr2d and Sprr2h did not occur (Figure 4). Surprisingly, there was also a significant increase without the presence of calcium, indicating that differentiation is not a requirement for Nrf2-induced expression of Sprr2d and Sprr2h in keratinocytes.

DISCUSSION

The process of replacing the keratinocyte plasma membrane with an insoluble matrix of crosslinked proteins and lipids enabled the evolutionary leap from an exclusively aquatic existence to sustainable life on land. Thus, CE proteins can be found in all living tetrapods, indicating their early evolution and requirement for terrestrial survival (Hohl et al., 1993).

This coincides with the conservation of Nrf2, especially among terrestrial animals (Maher and Yamamoto, 2010). Our data suggest that the conservation of Nrf2 may be partially due to its ability to sense a barrier defect in utero and orchestrate the upregulation of epidermal differentiation genes to ensure survival.

We limited our search for Nrf2-stabilizing molecules to metabolites in amniotic fluid, as we predicted that these small molecules could diffuse into keratinocytes within the developing epidermis. However, the diffusion of small molecules into keratinocytes in utero conflicts with the hypothesized role of the peri-derm layer. The periderm, a single-cell layer that sloughs from the surface of the developing epidermis once barrier formation occurs, has been previously hypothesized to serve as a temporary barrier during development (Hardman et al., 1998). Despite this hypothesized role, Toluidine Blue O, which has a MW of 308, can clearly pass through the periderm into the developing epidermis. Thus, the dye penetration assay suggests that other compounds of a similar weight, such as small molecule metabolites, could also pass through the periderm to trigger the compensatory response if barrier formation was delayed. This coincides with the compositional changes seen between E14.5 and E16.5 amniotic fluid. Furthermore, some of the metabolites identified only in E16.5 amniotic fluid have atomic compositions reminiscent of Nrf2-activating compounds.

Interestingly, the Nrf2/Keap1 pathway does not appear to play a role in maintaining an epidermal barrier after birth, as evidenced by the lack of a phenotype in K14ΔNrf2 mice (auf dem Keller et al., 2006) and our ΔNrf2 mice (this study). In fact, mice engineered to express Nrf2 constitutively in the epidermis have recently been shown to exhibit a severe ichthyosis, which was hypothesized to result from the sustained increase in expression and incorporation of Sprr2d and Sprr2h proteins into the CE (Schäfer et al., 2012). Collectively, these data suggest that the Nrf2/Keap1 pathway has evolved to quickly respond to failure to form a barrier; however, during homeostasis this pathway appears to be relegated to its more classically studied role in antioxidant defense (Schäfer et al., 2010).

Recent studies have also shown that some of the epithelial-specific genes induced by Nrf2 may have multifaceted roles in keratinocytes. Sprrs have been shown to not only incorporate into CEs but to also be transiently upregulated in stratified epithelia during a wound response to directly quench ROS via their highly reactive cysteine residues (Demetris et al., 2008; Vermeij and Backendorf, 2010). Based on these multifaceted properties, Sprr2 family members are perfectly suited to respond to different types of stress induced by barrier defects. In the LKO, Sprr2 proteins reinforce the CE; however, in ft/ft mice that lack filaggrin, Sprr2 proteins were mainly cytosolic, suggesting a role in scavenging electrophiles and reducing oxidative stress.

To confirm Nrf2 as the primary sensor and activator of the compensatory response in the LKO, we utilized a dominant-negative transgenic approach because of the existence of other Nrf family members that could compensate for the loss of Nrf2 (auf dem Keller et al., 2006). In addition to confirming the role of Nrf2 in orchestrating the compensatory response in the LKO, we demonstrated that the Nrf2/Keap1 pathway could be preemptively activated in the LKO in utero by small molecules. Therefore, our current focus is aimed at exploiting this mechanism in cases in which the barrier is compromised, such as accelerating barrier formation in premature infants and wounding in adults.

In summary, we have unveiled an unexpected mechanism that has evolved to detect a barrier defect in utero to ensure survival. Our results documenting that the Nrf2-regulated compensatory response is activated in embryos lacking both loricrin and filaggrin may explain why mice deficient in other CE components, including involucrin (Djian et al., 2000), envoplakin (Määttää et al., 2001), and periplakin (Aho et al., 2004), as well as

humans deficient in filaggrin (Smith et al., 2006), are born with either no or relatively mild barrier defects.

EXPERIMENTAL PROCEDURES

Primary Keratinocyte and Amniotic Fluid Isolation

Keratinocytes were isolated from newborn mice essentially as described (Lichti et al., 2008) with the following modifications: keratinocytes were plated on type IV collagen using defined keratinocyte serum-free medium (DKSFM; Invitrogen, Carlsbad, CA, USA) supplemented with a Rho-kinase inhibitor to facilitate initial plating and growth (Calbiochem, Darmstadt, Germany). For all experiments involving amniotic fluid, a 50/50 mixture of amniotic fluid and DKSFM was used. To collect amniotic fluid, a 22-gauge needle was inserted into each amniotic sac to extract the fluid. All collected amniotic fluid was then pooled, filtered through a 0.22 μm filter, and frozen at −80°C until use.

hPAP Histochemistry

Primary AREhPAP$^+$ keratinocytes were grown to 70% confluency and exposed to 1.2 mM Ca^{2+}, DMSO, 30 μM tBHQ, E14.5, E15.5, or E16.5 amniotic fluid for 24 hr. Following treatment, the cells were washed, fixed in 4% PFA, and incubated in TMN buffer heated to 65°C (pH 9.5) (50 mM Tris, 5 mM $MgCl_2$, and 100 mM NaCl). hPAP activity was determined by incubating in TMN buffer containing 1 mg/ml NBT and 1 mg/ml BCIP (Promega, Madison, WI, USA). The colorimetric reaction was stopped with H_2O and mounted with an aqueous mounting media (Vector Laboratories, Burlingame, CA, USA).

Immunofluoresence and Immunohistochemistry

Paraffin sections were deparaffinized and rehydrated, and antigen retrieval was performed by steaming in 10 mM sodium citrate buffer (pH 6.0). Sections were incubated with primary antibody overnight at 4°C. Presence of bound antibody was then determined using an HRP-conjugated secondary and the Vectastain ABC system and Nova-Red chromogenic substrate (Vector Laboratories) for immunohistochemistry, or Alexa Fluor secondary antibodies (Invitrogen) for immunofluoresence. For immunohistochemistry, slides were then counterstained with hematoxylin, dehydrated, and mounted with Permount.

Generation of Nrf2 Antibody

Commercial antibodies raised against Nrf2 were not able to detect endogenous Nrf2 in our studies (data not shown). Therefore, we generated our own affinity-purified rabbit anti-mouse Nrf2 antibody as previously described (Suvorova et al., 2009). Briefly, recombinant, Histidine-tagged, full-length mouse Nrf2 protein was purified and used to generate Nrf2 antisera in two rabbits. Anti-Nrf2 antibodies were then affinity purified using GST-tagged Nrf2.

Luciferase Constructs and Transfection

Sprr2d and Sprr2h AREs were identified using the sequence TGACnnnGC and Geneious bioinformatics software, version 4.8.5. We generated 50 bp oligomers bracketing each core ARE and ΔARE (Integrated DNA Technologies, Coralville, IA, USA) and cloned each of them into a pGL3 luciferase vector (Promega). Each of these constructs along with a pGL2-CMV-βgal was used for transfection of primary mouse keratinocytes by electroporation using the Amaxa human keratinocyte kit (Lonza, Basel, Switzerland), in accordance with the recommended protocol. Keratinocytes were plated and incubated with or without 30 μM tBHQ (Sigma-Aldrich, St. Louis, MO, USA) for 16 hr. Following tBHQ incubation, the keratinocytes were lysed and processed to measure luciferase activity using the Dual-Light

Combined Reporter Gene Assay System for detection of luciferase and beta-galactosidase (ABI) on a Glomax luminometer (Promega).

Chromatin Immunoprecipitation

Primary mouse keratinocytes were plated to 70% confluency on 15 cm dishes and treated with 1.2 mM $CaCl_2$ for 48 hr to induce differentiation. After differentiation, cells were exposed to tBHQ for 5 hr to induce nuclear translocation of Nrf2. Following tBHQ treatment, chromatin immunoprecipitation was performed using 10 μg of affinity-purified Nrf2 antibody and primers bracketing the Sprr2d, Sprr2h, and Nqo1 AREs.

Generation of ΔNrf2 Mice

cDNA encoding the ΔNrf2 was amplified by PCR with additional BamHI sites (underlined) added at each end using the oligonucleotides 5′-ATG CGG ATT C A TGC GTG AAT CCC AAT G-3′ and 5′-ATG CCC TAG GGG CTC CAT CCT CCC GAA C-3′. The PCR product was subcloned, sequenced, and inserted into a previously described loricrin expression vector (DiSepio et al., 1995). The ΔNrf2 and surrounding loricrin regulatory sequences were separated from the backbone and injected into the pronuclei of one-cell-stage FVB/N embryos. Expression of the transgene was determined by immunofluorescence.

Sulforaphane Treatment and Determination of Barrier Formation

Pregnant mice were injected i.p. with 20 μmol (*R,S*)-sulforaphane (SF) (LKT Labs, St. Paul, MN, USA) at E14.5 and E15.5, collected at E16.5, briefly dehydrated in methanol, and rehydrated in PBS. The embryos were then submerged in 0.0125% toluidine blue O for 1 min and immediately washed in PBS.

The Supplemental Experimental Procedures contain more complete methods, including those related to real-time PCR, western blot, luciferase reporter assay, and chromatin immunoprecipitation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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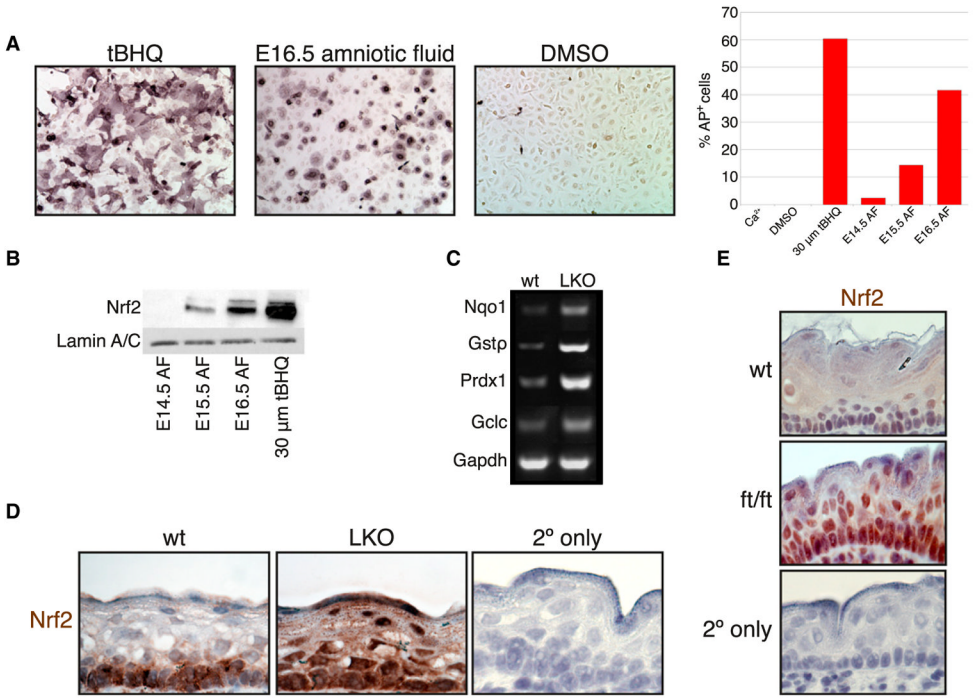

Figure 1. Exposure to Amniotic Fluid Activates Nrf2 In Vitro and In Vivo

(A) Keratinocytes were isolated from a transgenic mouse line containing an Nrf2-inducible hPAP reporter (AREhPAP) and exposed to tBHQ (left panel), E16.5 amniotic fluid (center panel), or DMSO (right panel). hPAP activity was detected by NBT/BCIP colorimetery. Graph shows the total percentage of cells stained positive for AP at each time point.

(B) Western blot for Nrf2 following nuclear extraction of primary keratinocytes after incubation with E14.5, E15.5, or E16.5 amniotic fluid for 16 hr.

(C) RT-PCR of RNA isolated from whole skin of E16.5 WT or LKO mice to detect the activation of Nrf2 downstream targets.

(D) Detection of Nrf2 by immunohistochemistry on E16.5 dorsal epidermis from WT and LKO mice. Right panel is 2° only control.

(E) Detection of Nrf2 by immunohistochemistry on E17.5 dorsal epidermis from WT and ft/ft mice. Bottom panel is 2° only control.

See also Figure S1 and Table S1.

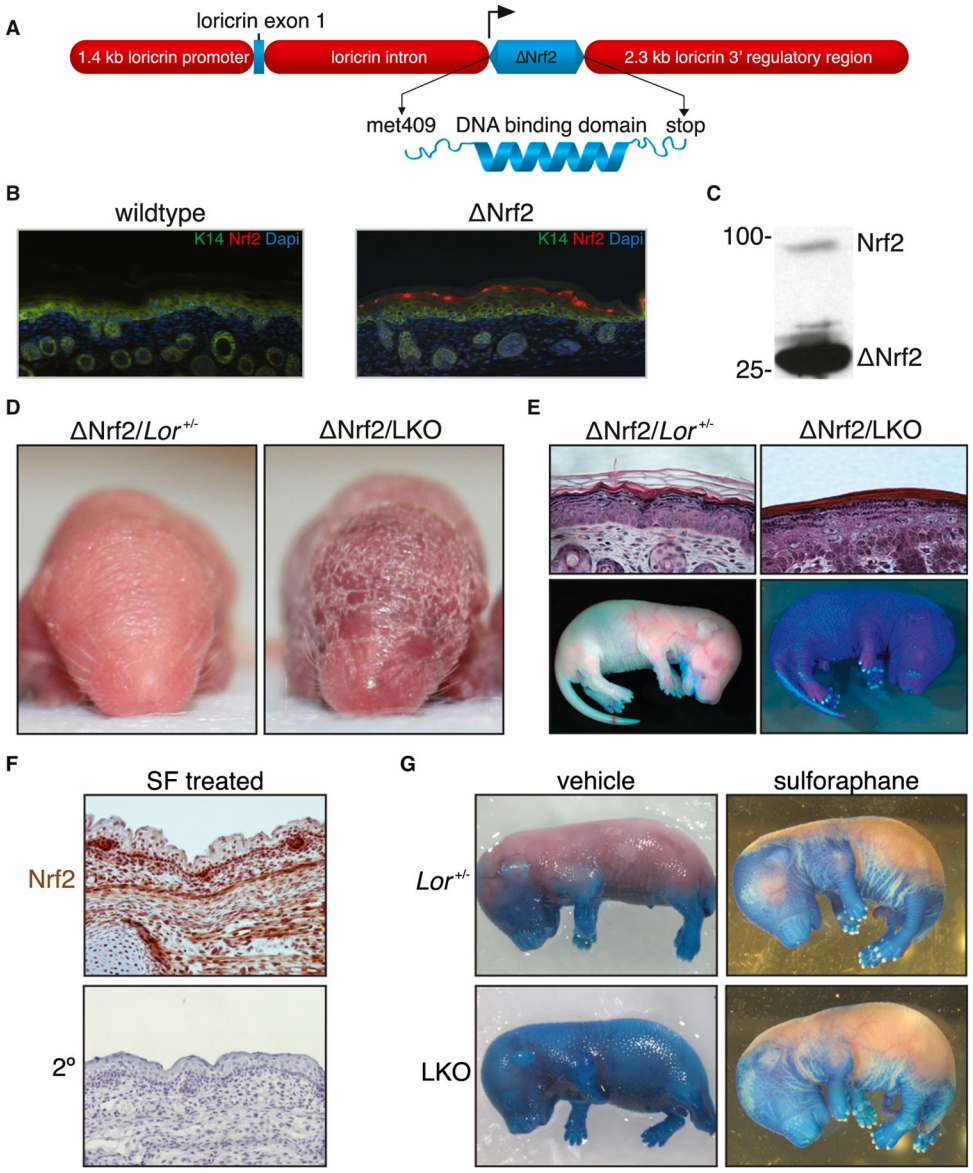

Figure 2. Nrf2 Orchestrates the Compensatory Response in the LKO

(A) Design of transgene used to generate ΔNrf2 mice.

(B) Nrf2 levels in both wild-type (left panel) and ΔNrf2^{+} mice (right panel) by immunofluoresence.

(C) Western blot from differentiated ΔNrf2^{+} keratinocytes comparing endogenous Nrf2 to ΔNrf2.

(D) Gross phenotype of newborn ΔNrf2/*Lor*$^{+/-}$ and ΔNrf2/LKO mice at postnatal day 0.

(E) Hematoxylin and eosin stained sections (upper panels) of newborn ΔNrf2/*Lor*$^{+/-}$ and ΔNrf2/LKO epidermis, highlighting a collapse of the stratum corneum (SC) layer in the ΔNrf2/LKO mice. To confirm a defect in the barrier of LKO/ΔNrf2 mice, toluidine dye penetration assay was performed on embryos extracted at E19.5 (bottom panels).

(F) Timed female mice were injected i.p. with SF at E15.5, and embryos were examined for Nrf2 stabilization by immunohistochemistry at E16.5.

(G) Injection of PBS or SF injected IP into $Lor^{+/-}$ females crossed with LKO males was performed at E14.5 and E15.5, and embryos were examined at E16.5 for barrier formation by toluidine blue O.
See also Figure S2.

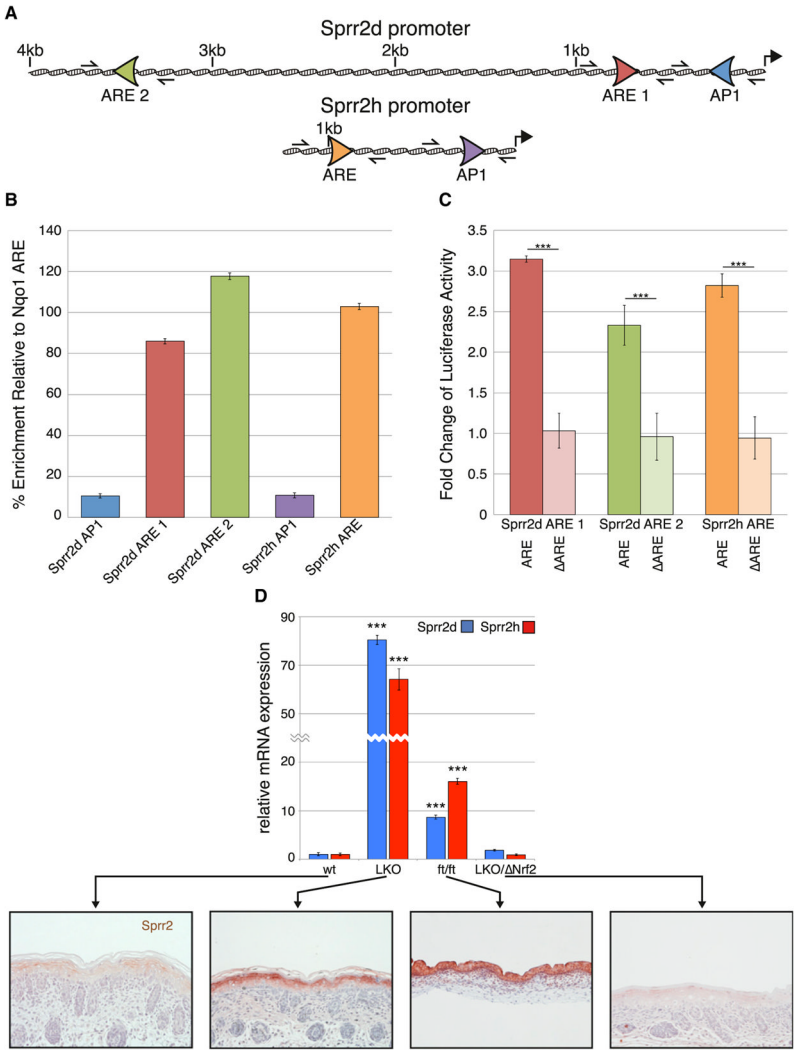

Figure 3. Sprr2d and Sprr2h Are Direct Downstream Targets of Nrf2

(A) Map of the Sprr2d and Sprr2h promoters. AREs in Sprr2d were found at −800 bp and −3.4 kb, and one ARE was found in the Sprr2h promoter at −950 bp. AP1 sites at −200 bp are also present. Arrows indicate the location of primers used for ChIP experiments.

(B) Nqo1 ARE enrichment was compared to the Sprr2d and Sprr2h AREs and AP1 sequences using ChIP.

(C) Reporter constructs containing each Sprr2d and Sprr2h ARE and ΔARE sites were generated. Primary keratinocytes were transfected with the labeled constructs and incubated with or without 30 uM tBHQ. Luciferase activity was measured, and the ratio of tBHQ-treated transfected keratinocytes to nontreated transfected keratinocytes was determined.

(D) Top graph shows the transcriptional upregulation of both Sprr2d and Sprr2h by real-time PCR in vivo at E16.5 in wild-type, LKO, and LKO/ΔNrf2 embryos and in E17.5 ft/ft embryos. Bottom panels correlate the amount of Sprr2 protein to the Sprr2d and Sprr2h transcript levels obtained for each mouse line by immunohistochemistry. *** indicates a $p < 0.001$ between keratinocytes transfected with ARE and ΔARE constructs in (C) and between each corresponding group to the WT expression in (D). Each experiment was performed on three individual samples run in triplicate, and the error bars represent the SD between replicates.

See also Table S2.

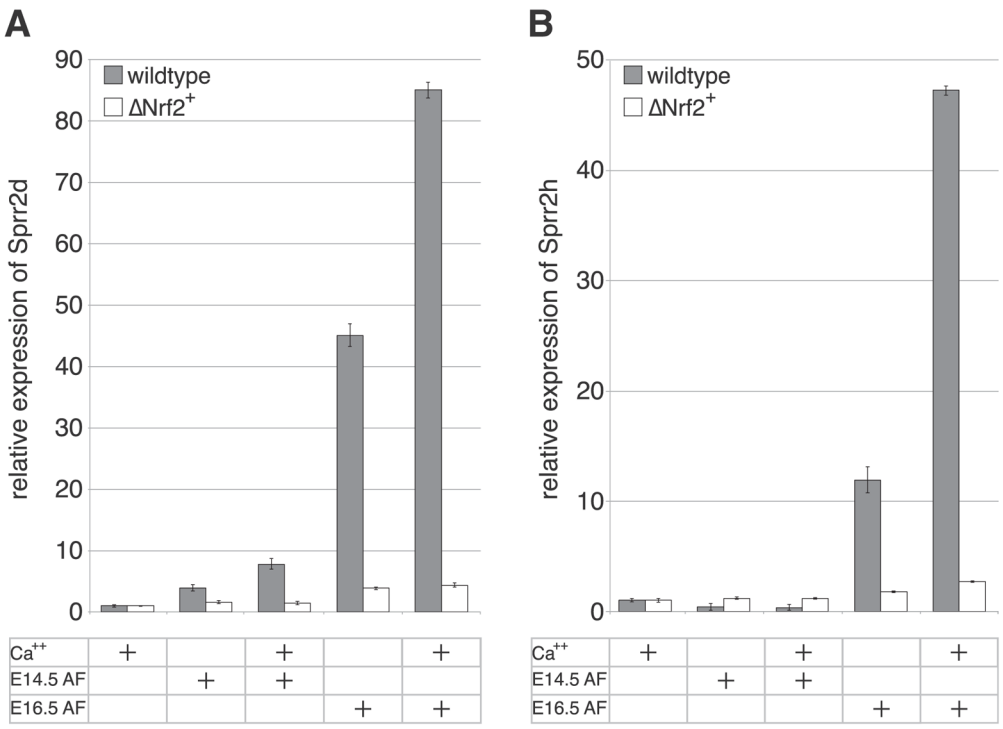

Figure 4. Keratinocytes Exposed to E16.5 Amniotic Fluid Upregulate Sprr2d and Sprr2h

(A) Sprr2d levels measured by real-time PCR using RNA from WT and $\Delta Nrf2^{+}$ keratinocytes exposed to Ca^{2+} and/or amniotic fluid.

(B) Sprr2h levels measured by real-time PCR using RNA from WT and $\Delta Nrf2^{+}$ keratinocytes exposed to Ca^{2+} and/or amniotic fluid. Each experiment was performed on three individual samples run in triplicate, and error bars represent the SD between replicates.