Reprogramming of keratin biosynthesis by sulforaphane restores skin integrity in epidermolysis bullosa simplex

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Epidermolysis bullosa simplex (EBS) is a rare inherited condition in which the epidermis loses its integrity after mechanical trauma. EBS is typified by the dysfunction of intermediate filaments in basal keratinocytes of epidermis. Most cases of EBS are due to mutations in the keratin 5 or 14 gene (*K5* and *K14*), whose products copolymerize to form intermediate filaments in basal keratinocytes. Available treatments for this disorder are only palliative. Here we exploit functional redundancy within the keratin gene family as the basis for therapy. We show that genetic activation of Gli2 or treatment with a pharmacological activator of Nrf2, two transcription factors eliciting distinct transcriptional programs, alleviates the blistering caused by a K14 deficiency in an EBS mouse model, correlating with K17 induction in basal epidermal keratinocytes. Nrf2 induction is brought about by treatment with sulforaphane, a natural product. Sulforaphane thus represents an attractive option for the prevention of skin blistering associated with K14 mutations in EBS.

Gli2 | keratin 14 null | keratin 17 | NQO1 | Nrf2

Epidermolysis bullosa (EB) refers to a group of diseases inherited with an incidence of ≈1:20,000 and is typified by the appearance of fluid-filled blisters after mechanical trauma to the skin (1) (Human Intermediate Filament Database, www.interfil.org). In the most frequent subtype of EB, called EB simplex (EBS), trauma-induced loss of skin integrity occurs within the epithelium (1). Most cases of EBS are due to dominantly acting mutations in keratin 5 or 14 (K5 and K14), which copolymerize into cytoskeletal intermediate filaments (IF) in basal keratinocytes of epidermis and other stratified epithelia (2–4). Treatment options for EBS, a debilitating and sometimes lethal disease, are only palliative. EBS is representative of a large number of tissue fragility conditions caused by inherited mutations in IF protein-encoding genes (5, 6).

Keratins belong to the large family of IF-forming proteins. The 54 known keratin genes are divided into 28 type I and 26 type II IF genes. Type I and II keratin proteins heterodimerize as they initiate polymerization into 10- to 12-nm-wide IF; beyond the resulting requirement for pairwise expression, type I and II keratin genes are regulated in a differentiation-dependent fashion *in vivo* (7). In addition to correctly hinting at the etiology of EBS, targeted expression of mutant K14 proteins revealed the key role of keratin IF toward mechanical support in basal keratinocytes of epidermis (8, 9). Since then, all types of IF have been implicated in structural support, and many of the >40 diseases linked to mutations in IF proteins involve cell fragility (6).

Given that many keratin proteins are structurally homologous, are long-lived, and exhibit overlapping distribution in epithelia, "redundancy" acts as a powerful modulator of the penetrance and severity of phenotypes caused by keratin dysfunction (10). In epidermis, basal cells transcribe the K5/K14 genes along with small amounts of K15, another type I keratin, whereas suprabasal keratinocytes transcribe the K1/K10 genes at an early stage, and the K2 gene at a later stage, of differentiation (7). K5 null mice (11) exhibit a more severe phenotype than K14 null animals (12), secondary to K15's ability to form a partially functioning filament network with K5 (13–15). Targeted expression of K16, but not K18, rescues K14 null mice from skin blistering, correlating with the greater homology between K14 and K16 (16, 17). The latter is significant because the induction of the wound repair-associated K6, K16, and K17 may account for the ability of EBS blisters to heal without scarring (9). Mice null for K10 fail to display fragility in the suprabasal layers of epidermis, correlating with the up-regulation of K5/K14 and K6/K16/K17 (18).

Current strategies for the treatment of keratin-based disorders include gene therapy (i.e., at the DNA level) or allele-specific silencing (mRNA level) (19, 20). A distinct strategy would involve an intervention at the protein level by triggering, for instance, the ectopic expression of functionally related keratins. Accumulation of the surrogate keratins can be predicted to attenuate the dominant-negative impact of a mutant protein (21, 22). Here we report on two signaling pathways, hedgehog and Keap1/Nrf2/ARE, which, upon activation, rescue skin blistering in an EBS mouse model, correlating with the reprogramming of keratin synthesis in epidermis. Our findings lead us to posit that natural chemicals able to selectively induce the Keap1/Nrf2/ARE pathway represent a promising option for the prevention of the pathological skin fragility occurring in EBS.

Results

Ectopic Expression of Gli2 Rescues Skin Blistering in $K14^{-/-}$ Mice. Keratin 17 (K17) is highly homologous to K14 (10) and a direct target for the transcription factor Gli2, a terminal effector of hedgehog signaling (23). The availability of $Gli2^{TG}$ mice, in which Gli2 expression is driven by the *K5* promoter (24), provided an opportunity to conduct a "proof-of-principle" experiment whereby sustained expression of $Gli2^{TG}$ in $K14^{-/-}$ mice should ameliorate oral and skin blistering secondary to the induction of K17 in basal keratinocytes. The skin of $Gli2^{TG}$ mice is normal for a few weeks after birth [see supporting information (*SI*) *Text*]. K17 protein occurs at higher levels (2.2-fold) in $Gli2^{TG}$ newborn skin compared with WT (SI Fig. 5*A*); levels of K5 are unchanged whereas those of K14 and K6 proteins are decreased (SI Fig. 5*A*). By immunostaining, K17 is robustly induced in the basal layer of $Gli2^{TG}$ interfollicular epidermis (SI Fig. 5*B*).

$Gli2^{TG}$ and $K14^{+/-}$ mice were crossed, and their progeny were

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Abbreviations: EB, epidermolysis bullosa; EBS, EB simplex; IF, intermediate filament; SF, sulforaphane; P*n*, postnatal day *n*.

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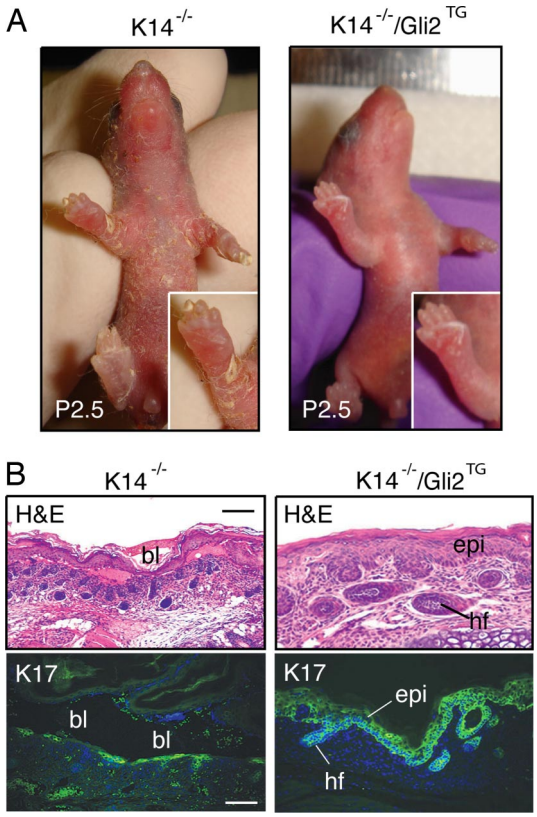

Fig. 1. Gli2-induced K17 expression rescues K14$^{-/-}$ mice. (*A*) Severe blistering on the forepaw of a P2.5 *K14*$^{-/-}$ mouse, which is dramatically reduced in a littermate *K14*$^{-/-}$/*Gli2*^{TG} mouse. (*B*) Comparing epidermal histology and biochemical markers in *K14*$^{-/-}$ and *K14*$^{-/-}$/*Gli2*^{TG} mouse tissue at P2.5. H&E-stained sections of forepaw skin show blistering (bl) in *K14*$^{-/-}$ mice but not in *K14*$^{-/-}$/*Gli2*^{TG} mice. There is a marked up-regulation of K17 in the epidermis of *K14*$^{-/-}$/*Gli2*^{TG} mice. bl, blister; epi, epidermis; hf, hair follicle. (Scale bars: 100 μm.)

analyzed. Unlike *K14*$^{-/-}$ mice, all *K14*$^{-/-}$/*Gli2*^{TG} mice were viable and showed normal skin ($n = 36$) (Fig. 1). The epidermis was intact and histologically normal, and basal keratinocytes were strongly immunoreactive for K17 (Fig. 1*B*). A small subset of *K14*$^{-/-}$/*Gli2*^{TG} mice showed skin fragility restricted to a small area in the ventral/posterior aspect of the front paws (Fig. 1*A Right Inset*), suggesting some degree of epidermal fragility in areas of highest frictional trauma. *Gli2*^{TG} mice were also mated with *K5*$^{+/-}$ mice as a control experiment. *K5*$^{-/-}$/*Gli2*^{TG} mice died shortly after birth and exhibited extensive blistering just like *K5*$^{-/-}$ mice ($n = 18$) (SI Fig. 6). Although the sustained activation of hedgehog signaling over time is not clinically desirable because it is oncogenic (23, 24), these findings establish that activation of a suitable signaling pathway can rescue the epithelial fragility characteristic of EBS.

Sulforaphane (SF) Selectively Induces K17 and K16 in Skin Keratinocytes. Inactivation of the *keap1* gene, whose protein product sequesters the transcription factor Nrf2 in the cytoplasm, targeting it for ubiquitination and degradation, causes esophageal hyperkeratosis paralleled by an up-regulation of K6 but no changes in K14 levels (25). We tested whether activation of Nrf2 impacts keratin expression in a similar fashion in skin keratinocytes. To do so we used the isothiocyanate SF, a natural product (ref. 26; see *Discussion*). SF activates Nrf2-dependent transcription secondary to its ability to modify Keap1 and prevent Nrf2 degradation (27). Cultures of 308 mouse skin keratinocytes were treated with 1 μM SF (in acetonitrile), and keratin mRNA levels were assessed by RT-PCR at 12 and 24 h after treatment. SF-treated cells show a significant increase in the mRNA level for NQO1, an established SF target (28). K17 and K16 mRNAs were elevated ≈2.5-fold at 12 h after treatment, but their levels returned to baseline by 24 h. K6a and K5 mRNAs showed only a slight induction, whereas the K6b, K14, and K15 mRNAs were unaltered (Fig. 2*A*). Immunolabeling revealed an obvious induction of K17, but not K14, at the protein level (Fig. 2*B*). At higher doses, SF induces apoptosis in many cell types (reviewed in ref. 29). We found that 1 μM SF does not trigger apoptosis in newborn mouse keratinocytes, whereas 5 μM SF does (94% of SF-treated cells versus 16% in control).

To uncover whether SF had similar effects *in vivo* and after sustained treatment, SKH-1 hairless mice were topically treated with 1 μmol of SF in jojoba oil twice a week for 4 weeks. Jojoba oil readily penetrates the skin (30) and does not induce Nrf2 activity. Relative to vehicle-treated skin, SF-treated skin shows markedly increased K17 immunoreactivity that extends to the basal layer of the epidermis. K16 immunoreactivity was also increased (data not shown), whereas K14 showed no change (Fig. 2*C*). Analysis of total skin protein extracts demonstrated increased levels of K17 and K16 in SF-treated skin, whereas again the level of K14 was unaltered (Fig. 2*D*). This sustained topical treatment regimen did not affect skin morphology (Fig. 2*C*) or alter the rate of apoptosis (data not shown), consistent with a previous study involving daily treatment with SF over an 11-week period (28).

Optimization of the SF Treatment Regime for *K14*$^{-/-}$ Mice. We next tested whether SF treatment could reduce skin blistering in *K14*$^{-/-}$ mice. Although the fragility of epidermal basal cells and associated trauma-induced cutaneous blistering of *K14*$^{-/-}$ mice mimic EBS as seen in humans (12), this model presents several challenges not relevant to most EBS patients. One key limitation is lethality, a likely consequence of severe oral blistering (SI Fig. 7). The mean survival of *K14*$^{-/-}$ mice was 2.5 ± 0.35 days ($n = 14$); by that time, they were smaller than controls, lacked milk in their stomachs, and were unable to close their mouths because of swelling. By comparison, lethality and severe oral lesions are less frequently seen in human newborns afflicted with EBS (1). The *K14*$^{-/-}$ mouse model thus provides a stringent test of the notion that SF could be effective as a treatment for EBS.

Initially, *K14*$^{-/-}$ mice received topical applications of 1 μmol of SF in jojoba oil at postnatal day 0 (P0), P1, and P3. This regime reduced cutaneous blistering in *K14*$^{-/-}$ pups, although inconsistently. Follow-up analyses showed that *K14*$^{-/-}$ mice already exhibited skin blistering at birth (Fig. 3*C*), i.e., before the first SF application. In *K14*$^{-/-}$/*Gli*^{TG} mice and in previous instances of rescue (17), expression of the "replacement" keratin (K17 and K16, respectively) began well ahead of birth. Given evidence that SF may cross the placental barrier (31), we posited that systemic treatment of pregnant mothers would cause prenatal induction of K16/K17 in fetal epidermis. To test this notion, pregnant females received an i.p. injection of 5 μmol of SF on the last day of pregnancy; we retrieved the embryos 2 h later, and the levels of dithiocarbamates were assayed as described above. We found that dithiocarbamates were readily detectable in the embryos at 2 h after treatment. The levels of dithiocarbamates in embryonic skin were 3.64 ± 1.17 nmol/mg protein, i.e., slightly higher than in the rest of the body (2.82 ± 0.66 nmol/mg protein). Analysis of P1 mice born from mothers that had received 5 μM SF i.p. showed, 24 h after birth, a clear induction of K17 protein in epidermis and oral mucosa (Fig. 3 *A* and *B*). *K14*$^{-/-}$ newborns exposed to SF *in utero* had markedly less blistering of their forepaws, back skin, and snout than untreated controls (Fig. 3*C*). Consequently, we devised a new treatment regime consisting of three i.p. injections of 5 μmol of SF to the pregnant mother every

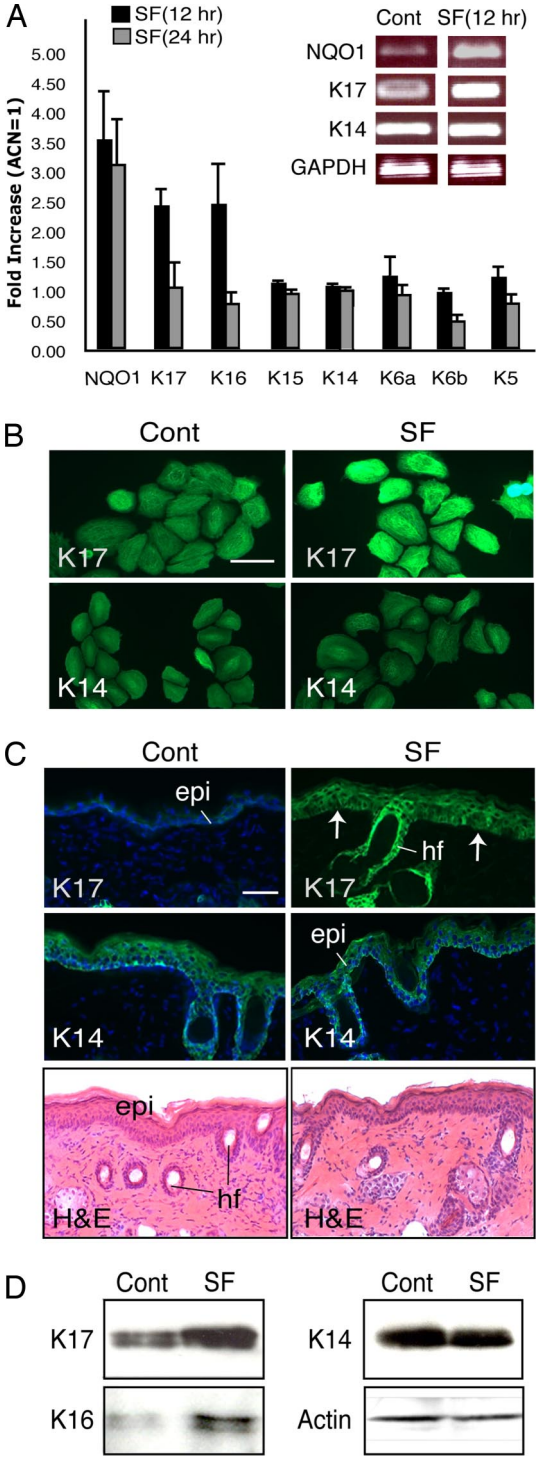

Fig. 2. SF induces K16 and K17 in skin keratinocytes. (*A* and *B*) Cultures of skin keratinocytes were treated with 1 μM SF in acetonitrile vehicle. (*A*) Relative levels of key mRNAs at 12 and 24 h after treatment, relative to acetonitrile-treated controls, and normalized to baseline. GAPDH serves as internal control. Results (mean ± SEM) are from three experiments. Representative results are shown in the upper right corner. (*B*) Immunostaining for K17 and K14 in acetonitrile-treated (Cont) and SF-treated cells 24 h after treatment. (*C* and *D*) Adult SKH-1 hairless mice were topically treated on their backs with 1 μmol of SF in jojoba oil (100 μl) or jojoba oil alone (Cont) for 4 weeks. Treated tissue was harvested 5 days after the last application and processed for morphological analyses (*C*) or protein extraction, electrophoresis, and Western blotting (*D*). In *D*, levels of K17, K16, and K14 antigens are assessed relative to actin. epi, epidermis; hf, hair follicle. (Scale bars: 100 μm.)

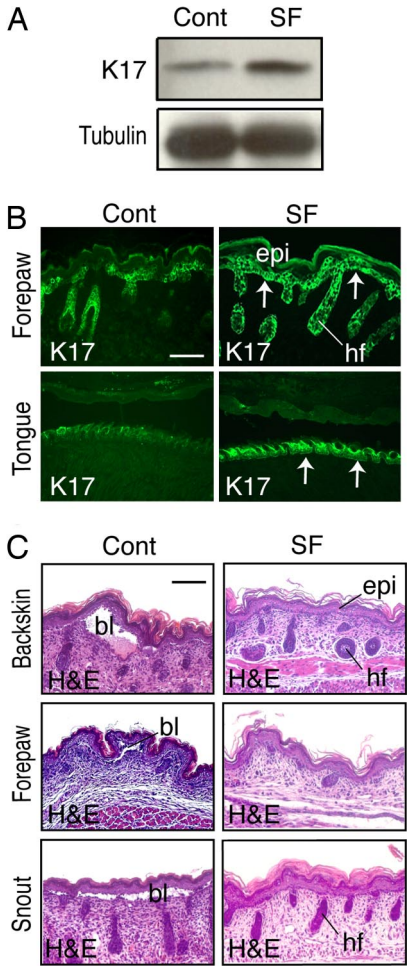

Fig. 3. SF treatment of pregnant $K14^{-/-}$ mothers causes induction of K17 in fetal epidermis. Tissue from newborn mice was harvested 24 h after the mothers received the last of three injections of 5 μmol of SF or vehicle, or were untreated, and evaluated by protein extraction and analysis (*A*), indirect immunofluorescence (*B*), or H&E staining (*C*). In *A*, tubulin is the loading control. In *B*, arrows denote induced K17 in SF-exposed tissues. In *C*, blistering (bl) is seen in control $K14^{-/-}$ null pups but not in $K14^{-/-}$ pups born from SF-treated mothers. The tissue source is indicated at left. epi, epidermis; hf, hair follicle. (Scale bars: 100 μm.)

other day in the week before delivery, and topical application of 1 μmol of SF (in jojoba oil) to the newborn at P0, P1, and P3.

SF Treatment Greatly Reduces Cutaneous Blistering in $K14^{-/-}$ Mice. The revised treatment regimen had a dramatic and consistent effect on the appearance and skin integrity of $K14^{-/-}$ mice. $K14^{-/-}$ pups could no longer be identified based on their physical appearance and behavior at P0.5 (Fig. 4 *A*, *A′*, *B*, and *B′*) and P2.5 (Fig. 4 *C*, *C′*, *D*, and *D′*). At P4.5, many of the $K14^{-/-}$ pups showed limited blistering restricted to the front paws (Fig. 4 *E*, *E′*, *F*, and *F′*). During that period, the difference between untreated and SF-treated $K14^{-/-}$ pups was striking (Fig. 4 *A*–*F* and *A′*–*F′*). Whereas 93% of the untreated $K14^{-/-}$ pups were dead by P3 (n = 14), 85% of the SF-treated $K14^{-/-}$ pups (n = 20) were alive at P4 (SI Table 1). Because $K14^{-/-}$ pups treated only with oil (n = 6) exhibited unusually severe blistering resulting in complete loss of the epidermis on the forepaws and earlier death (67% dead by P2, probably because of increased manipulation), age-matched untreated $K14^{-/-}$ mice were used as controls in all subsequent studies.

Histological analyses were conducted with a focus on forepaw,

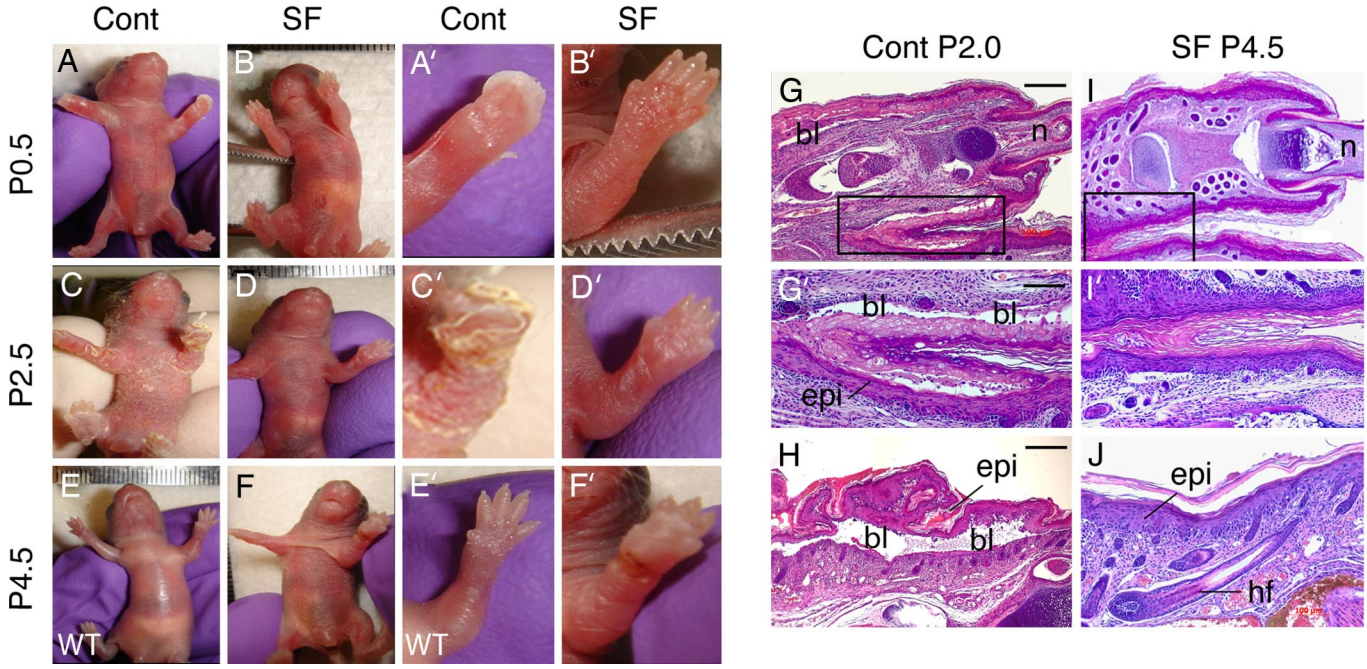

Fig. 4. SF treatment reduces skin blistering in newborn K14 null mice. (*A–F* and *A′–F′*) Pups receiving either no treatment (Cont) or SF shown at P0.5, P2.5, or P4.5. At P4.5, a wild-type (WT) mouse is shown for comparison (*E* and *E′*). (*A′–F′*) Detailed views of forepaw, an area prone to blistering. (*G–J*) Forepaws were surgically removed from P2 mice and processed for embedding, sectioning, and H&E staining. Micrographs are from digits in *G* and *I* and from dorsal forepaw skin in *H* and *J*. Boxed areas in *G* and *I* are detailed in *G′* and *I′*. bl, blistering; epi, epidermis; hf, hair follicle; n, nail. (Scale bars: 100 μm.)

which consistently shows severe skin blistering in $K14^{-/-}$ mice (Fig. 4 *A′* and *C′*). At P2.5 (data not shown) and P4.5 (Fig. 4 *G–J*) the epidermis was significantly protected in SF-treated mice relative to controls (P2 $K14^{-/-}$ mice). We determined the fraction of blistered skin per unit length of forepaw epidermis for untreated and treated $K14^{-/-}$ mice at P2 (*SI Text*). Treated $K14^{-/-}$ mice had only 23 ± 14% ($n = 6$) of blistered skin whereas untreated $K14^{-/-}$ mice had 85 ± 12% ($n = 6$ per group; $P < 0.05$). Reduction in blistering correlated with the presence of K17 in the basal layer of SF-treated epidermis. There also appeared to be an induction of K16, albeit to a lesser extent (SI Fig. 7). This contrasts with untreated $K14^{-/-}$ skin, which showed a spotty distribution of K17 and K16 restricted to the suprabasal compartment (SI Fig. 7). In addition, epidermal differentiation, as reflected by established markers, was normal in SF-treated $K14^{-/-}$ mice (SI Fig. 7), extending previous findings (see Fig. 2 and ref. 29).

Many $K14^{-/-}$ pups (P0) born from SF-treated mothers exhibited reduced oral blistering (SI Fig. 8), coinciding with increased K17 expression in the basal layer of tongue (Fig. 3). However, the topical mode of SF delivery during the postnatal phase of treatment was not effective for maintenance of K16/K17 expression in the oral mucosa (data not shown). Accordingly, many of the SF-treated $K14^{-/-}$ mice began to show signs of progressive wasting beyond P4 (SI Table 1), and most of them were dead by P6. Unlike skin, which was largely blister-free at that time (Fig. 4), there was severe blistering in the lips and oral mucosa (SI Fig. 8). Attempts to circumvent this limitation through systemic administration of SF to $K14^{-/-}$ pups were unsuccessful.

Applying the same prenatal and postnatal regime of SF treatment failed to rescue the severe skin-blistering phenotype, and death, of $K5^{-/-}$ pups (SI Table 1), suggesting that the induction of K17/K16 plays a significant role in the SF-mediated rescue of $K14^{-/-}$ pups.

Discussion

Local activation of two distinct signaling pathways, hedgehog and Keap1/Nrf2/ARE, resulted in a dramatic reduction of the skin blistering associated with a K14 deficiency in mouse (see SI Table 1). Because there is little commonality between the transcriptional programs set forth by hedgehog, a pro-growth signal (32), and Keap1/Nrf2/ARE, a pathway eliciting broad metabolic and antioxidant cellular responses (33), their shared ability to induce K17 (and K16 in the case of SF) in basal epidermal keratinocytes probably plays a significant role in this rescue. This is further supported by the inability of both Gli2 and SF to rescue any aspect of the $K5^{-/-}$ phenotype (SI Table 1) and the strong evidence pointing to functional redundancy in the keratin family (10). SF also has potent antiinflammatory properties (28, 34) that are manifested in the skin of treated $K14^{-/-}$ newborns (data not shown). This antiinflammatory effect may help limit the extent of tissue damage and/or assist tissue repair in $K14^{-/-}$ mouse skin (35). Altogether, these results corroborate the principle that functional redundancy within the keratin gene family can be exploited as the basis for therapy in EBS and related conditions.

The extent of Gli2-mediated phenotypic rescue of the $K14^{-/-}$ mouse strain is longer-lasting than with SF (consider that Gli2 expression is driven by the *K5* promoter, ensuring early and sustained expression in relevant epithelia, and the K17 promoter is a highly sensitive target of Gli2) (23). Although the complications associated with sustained Gli2 activity raise concerns about its use in a clinical setting, testing this pathway yielded key conceptual evidence supporting our proposal for EBS therapy. By comparison, the delivery of SF (which is of dietary origin) as an Nrf2 inducer could be under medical supervision and is suitable for use in humans.

SF was identified as the principal and potent inducer of NQO1 derived from broccoli (26). Some cultivars of brassica vegetables (e.g., broccoli sprouts and seeds) contain high concentrations of glucoraphanin, a precursor of SF that is hydrolyzed by the plant enzyme myrosinase upon maceration or by the intestinal microflora to yield the active SF (36). The inducer and protective effects elicited by SF require Nrf2 (37). Whether the induction of K17 and K16 in SF-treated skin also proceeds via a Nrf2-

dependent mechanism remains to be investigated (see *SI Text*). Broccoli sprout-derived extracts rich in SF are well tolerated when given orally to human subjects (38), and their topical application is effective at inducing Nrf2 target gene expression in healthy mouse and human skin (39). By virtue of its impact on phase 2 metabolism and antioxidant responses, sustained activation of Nrf2 activity may provide additional benefits to the skin, a tissue broadly exposed to the environment (39). We predict that SF will induce K16/K17 in human skin (see *SI Text*).

Our findings may be directly relevant to the small subset of EBS patients whose genome bears the equivalent of a K14 null mutation (Human Intermediate Filament Database, www.interfil.org). However, most cases of EBS are due to dominantly acting, missense alleles altering the function of either K14 or K5 (Human Intermediate Filament Database, www.interfil.org) (5, 6). Still, the available evidence suggests that Nrf2 inducers like SF would be therapeutically effective in EBS patients harboring any type of mutation at the K14 locus. First, the severe impact of large deletion mutants (in K14) on keratin assembly *in vitro* is attenuated by titrating in wild-type K14 protein (21). Second, the dominant-negative impact of a R_{125}→C allele of K14, associated with severe EBS disease in humans (2, 40), is attenuated and even "silenced" by activity of the intact K14 allele in transgenic mouse skin (22). Third, keratin IF organization appears surprisingly normal in cultures of keratinocytes isolated from EBS patients (41–43), correlating with expression of K6, K16, and K17 in this setting. Finally, the nonscarring healing of skin blisters in EBS patients is probably mediated in part by the induction of K6/K16/K17 (e.g., ref. 9). Because of its limited impact on K6 expression in epidermis, SF and other Nrf2 inducers should be ineffective in EBS cases caused by K5 mutations. On the other hand, SF and other Nrf2 inducers could possibly be beneficial in the treatment of other conditions in which the skin exhibits fragility as a result of a mutation in a gene encoding a key cytoskeletal component.

The effects of Nrf2 inducers on cellular protection are long-lived and typically outlast their impact on ARE-dependent transcription (44). We found that SF-induced K17 protein is very long-lived in mouse epidermis (SI Fig. 6). Thus, frequency of treatment with SF or a comparable Nrf2 inducer in a hypothetical clinical setting for EBS should be dictated by local determinants such as the amount of physical stress experienced and epidermal turnover rate. More frequent topical applications, or systemic treatment, may be required to achieve adequate protection during the first few months after birth, when the disease is more severe (1). These and other issues need to be addressed in pilot studies involving human subjects.

Methods

Animals, Antibodies, and Preparation of SF. All studies involving mice were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. SKH-1 hairless mice were purchased (Charles River Laboratories, Wilmington, MA). $K14^{-/-}$, $K5^{-/-}$, and $Gli2^{TG}$ mice were maintained as described (11, 12, 24). Antibodies used included rabbit polyclonals against K6, K16, or K17 (P.A.C. laboratory); K14, filaggrin, and K1 (Covance, Denver, PA); and a mouse monoclonal anti-actin (Sigma, St. Louis, MO). Fluorochrome-conjugated goat anti-rabbit antibodies were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). SF [1-isothiocyanato-4(*R*,*S*)-(methylsulfinyl)butane] was obtained from LKT Labs (St. Paul, MN). Solutions of SF in acetonitrile or jojoba oil (MP Biomedicals, Solon, OH) were prepared on the day of application.

Mouse and Cell Culture Studies. Pregnant $K14^{+/-}$ females were given approximately three i.p. injections of 5 μmol of SF in PBS every other day in the week before delivery. Neonates were each treated topically with 1 μmol of SF in jojoba oil (100-μl volume) on the day of birth (P0) and on P1 and P3 for 30 min. For determination of SF delivery to the fetus, a pregnant female was treated with SF (5 μmol in 25 μl of PBS) i.p. After 2 h, the mother was killed, embryos were harvested and washed, and the skin was separated from the rest of the body. Samples were frozen, stored at −80°C, and processed as described above. For chronic application studies, 7-week-old female SKH-1 hairless mice were topically treated twice a week for 4 weeks with either 1 μmol of SF in jojoba oil (100-μl volume) or jojoba oil vehicle and killed. Dorsal skin was harvested for analysis. Blood samples were also collected to test for levels of SF and metabolites as described above. Liver homogenates were analyzed for NQO1 enzyme activity (28, 39). $K14^{-/-}/Gli2^{TG}$ and $K5^{-/-}/Gli2^{TG}$ mice were killed at P2 and P0, respectively, and tissues were analyzed as described. 308 mouse epidermal keratinocytes were a gift of Stuart Yuspa (National Cancer Institute, Bethesda, MD). Indirect immunofluorescence was performed on cells fixed (4% paraformaldehyde) 12 h after treatment with 1 μM SF in acetonitrile or acetonitrile alone (0.1% concentration in medium).

Biochemical and Morphological Analyses. 308 cells were treated with media containing 1 μM SF in acetonitrile or acetonitrile alone. Cells were harvested at 12, 24, and 48 h later, and total RNA was extracted by using TRIzol (Invitrogen, Carlsbad, CA). After DNase treatment (RNase free DNase kit; Qiagen, Valencia, CA), 1 μg of total RNA from each time point was reverse-transcribed (Advantage RT-for PCR kit; Clontech, Mountain View, CA). First-strand cDNA was then used for PCR analysis of gene expression using Invitrogen *Taq*. Target mRNAs and oligonucleotide primer sets are given in SI Table 2. Intensity of electrophoresed, ethidium bromide-stained PCR products was quantified by using NIH Image. For protein analyses, dorsal skin was harvested from control and treated SKH-1 hairless mice, $K14^{-/-}/Gli2^{TG}$ mice, or $K5^{-/-}/Gli2^{TG}$ mice, and total protein was extracted (17). Protein concentrations were obtained by using a Bradford Assay Kit (Bio-Rad, Hercules, CA). Equal amounts of protein (10–20 μg) were resolved on 8.5% SDS/PAGE gels and blotted onto nitrocellulose. Bound primary antibodies were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech, Pittsburgh, PA) or alkaline phosphatase (Bio-Rad). For morphological analyses, tissues were fixed in Bouin's solution and paraffin-embedded, and 5-μm sections were cut and stained with H&E for routine histopathology or incubated with primary antibodies and revealed by indirect immunofluorescence using FITC-conjugated secondary antibodies. TUNEL staining was performed on postfixed (4% paraformaldehyde) fresh-frozen sections (Dead End Kit; Promega, Madison, WI).

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