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Sexual dimorphism in response to a NRF2 inducer in a model for pachyonychia congenita

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Abstract

Sex is an influential factor regarding pathophysiology and therapeutic response in human disease. Pachyonychia congenita (PC) is caused by mutations in keratin genes and typified by dystrophic lesions affecting nails, glands, oral mucosa, and palmar-plantar epidermis. Painful palmar-plantar keratoderma (PPK) severely impair mobility in PC. Mice genetically null for keratin 16 (*Krt16*), one of the genes mutated in PC, develop PC-like PPK. In male *Krt16*$^{-/-}$ mice, oxidative stress associated with impaired glutathione synthesis and NRF2-dependent gene expression precedes PPK onset, which can be prevented by topical sulforaphane (SF)-mediated activation of NRF2 (Kerns et al., J. Clin. Invest. 126:2356-66). We report here that SF treatment fails to activate NRF2 and prevent PPK in female *Krt16*$^{-/-}$ mice despite a similar set of molecular circumstances. Follow-up studies reveal a temporal shift in PPK onset in *Krt16*$^{-/-}$ females, coinciding with sex-specific fluctuations in footpad skin glutathione levels. Dual treatment with SF and diarylproprionitrile (DPN), an estrogen receptor beta (ER-β) selective agonist, results in NRF2 activation, normalization of glutathione levels, and prevention of PPK in female *Krt16*$^{-/-}$ mice. These findings point to a sex difference in NRF2 responsiveness that needs be considered when exploring NRF2 as a therapeutic target in skin disorders.

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Introduction

We recently reported that the PC-like PPK lesions arising in the paw skin of *Krt16*$^{-/-}$ mice are preceded by profound oxidative stress that results from impaired synthesis of the antioxidant glutathione secondary to hypo-functional nuclear factor erythroid-derived 2 related factor 2 (NRF2) (Kerns et al., 2016). The latter, a basic leucine zipper (bZIP) transcription factor that plays essential and pleiotropic roles in cellular responses to stress and maintenance of redox balance (Wang et al., 2013), is expressed at elevated levels but is hypo-phosphorylated and dysfunctional in *Krt16*$^{-/-}$ paw skin and in biopsies of PPK lesions in individuals with PC (Kerns et al., 2016). Topical treatment with sulforaphane (SF), a small molecule activator of NRF2 (Zhang et al., 1992), leads to restoration of the redox balance and prevention of PPK development in *Krt16*$^{-/-}$ paw skin (Kerns et al., 2016). Though it yielded novel insight into the pathophysiology of PC-associated PPK with significant implications for therapy, this previous study was restricted to male mice. Given emerging evidence of sexual dimorphism in several disease settings involving oxidative stress (Candeias et al., 2016, Cole et al., 2016, Dimitrijevic et al., 2016, Hanna et al., 2016, Pitts et al., 2015, Tostes et al., 2016) and in pharmacological response (Anderson et al., 2008), the question arises as to whether female *Krt16*$^{-/-}$ mice show a NRF2 responsiveness similar to their male counterparts.

Results and Discussion

At four weeks of age, i.e., immediately prior to the onset of PC-like PPK lesions in front paw skin, key morphological and molecular attributes in female *Krt16*$^{-/-}$ mice are comparable to those in male. Specifically, the levels of reduced glutathione (GSH; Figure 1A, Supplemental Table 3) and of the mRNA transcripts for glutamate-cysteine ligase (GCLC) and glutathione synthetase (GS; Figure 1B), enzymes required for GSH synthesis (Dalton et al., 2000), are decreased in female *Krt16*$^{-/-}$ paw skinrelative to wildtype (*WT*) control, indicating a state of oxidative stress resulting in part from impaired GSH synthesis. Paradoxically, *NRF2* is significantly upregulated in female *Krt16*$^{-/-}$ samples at the mRNA level (Figure 1C) and when assessed by indirect immunofluorescence in paw skin tissue sections (Supplemental Figure 1A). Albeit elevated, this pool of NRF2 is dysfunctional as can be inferred from the decreased mRNA levels for several of its key target genes, including GS, GCLC (Figure 1B), heme oxygenase 1 (HO-1), and NAD(P)H Quinone Dehydrogenase 1 (NQO-1; Figure 1C). Furthermore, relatively weak immunostaining is observed for the phosphorylated (activated) form of NRF2 and for two key upstream regulators of NRF2 activation, PKC-δ and RACK1, in tissue sections from female *Krt16*$^{-/-}$ mice compared to control (Supplemental Figure 1). Similar to the situation already reported for males (Kerns et al., 2016), therefore, female *Krt16*$^{-/-}$ paw skin shows clear signs of oxidative stress secondary to hypoactive NRF2 signaling ahead of lesion onset. Again, and as previously reported for male *Krt16*$^{-/-}$ mice (Kerns et al., 2016), the presence of copious amount of NRF2 protein offers an ideal opportunity for preventive therapy via topical SF in female *Krt16*$^{-/-}$ mice. Such a therapeutic approach for PC, regardless of the sex of the individual, is buttressed by the finding of elevated, but hypoactive NRF2 in the lesional plantar epidermis of both male and female PC patients (Supplemental Figure 2) (Kerns et al., 2016).

Cohorts of four-week old *Krt16*$^{-/-}$ females were subjected to a twice-weekly topical treatment regimen with the NRF2 inducer SF for four consecutive weeks, and harvested for analysis. Littermate male mice were included as a positive control for treatment outcome (Kerns et al., 2016). Interestingly, *Krt16*$^{-/-}$ males treated with jojoba oil (vehicle) exhibited significantly more severe lesions than vehicle-treated *Krt16*$^{-/-}$ females (Figure 1D and 1E), a phenomenon that may be secondary to oil-induced changes in the skin barrier and/or sensory nociception. Surprisingly, and in stark contrast to males, SF treatment had little effect on lesion development in *Krt16*$^{-/-}$ females (Figures 1D), as corroborated by measurement of a PPK quantitative index (Figure 1E) (see Methods and Kerns et al., 2016) and histological analysis (Figure 1F). Consistent with these findings, SF treatment failed to activate NRF2 in *Krt16*$^{-/-}$ female paw tissue relative to male counterparts, as conveyed by indirect immunofluorescence for total and phosphorylated NRF2 (Figure 1G; quantification in Supplemental Figure 3A and B).

The sexual dimorphism in the response to SF prompted us to more closely examine the *Krt16*$^{-/-}$ mouse model. First, we found no difference in the severity of early or late PPK-like lesions between male and female *Krt16*$^{-/-}$ mice (Figure 2A and 2B). Next, we compared the timing of lesion onset in male and female *Krt16*$^{-/-}$ mice. In both sexes, the earliest signs of paw lesion occur at 4 weeks and reach a peak at 6 weeks post-birth with no statistical difference (p value = 0.17) found between the average age of onset for males and females (33.4 ± 1.7 versus 36.4 ± 1.2; average + SEM) or the overall distributions of the two groups (Figure 2C; log-rank (Mantel-Cox) p value = 0.24). This said, a ~1.5 week lag of PPK lesion onset in females relative to males occurs within that 2-week interval, with the relative risk of lesion onset being ~2 to 3 fold less in female *Krt16*$^{-/-}$ mice from 4 to 5 weeks of age (Supplemental Table 1). We also analyzed glutathione levels in footpad skin of male and female mice aged 4 to 8 weeks. A significant sex-based difference was noted in oxidized glutathione (GSSG) levels, with males showing a transient increase at 5 weeks post-birth and females manifesting that surge slightly later, at 5.5 weeks post-birth (Figure 2D, Supplemental Table 2 and 3). By contrast, across this period, the levels of reduced glutathione (GSH) appeared relatively steady in both sexes (Figure 2E, Supplemental Table 2 and 3). The resulting GSH/GSSG ratio, which reflects the prevailing cellular redox balance (Pastore et al., 2001), was significantly higher in female mice until a drop at 5.5 weeks post-birth (Figure 2F, Supplemental Table 2 and 3), correlating with the later onset of PPK in *Krt16*$^{-/-}$ females. Similar fluctuations occurred in *WT* and *Krt16*$^{-/-}$ mice across the time frame surveyed, suggesting that sex-specific differences, rather than *Krt16* expression, underlie the slight delay of lesion onset in *Krt16*$^{-/-}$ females. Thus, there appear to be two components contributing to the increased levels of oxidative stress in the *Krt16*$^{-/-}$ mouse model, namely a sex-independent effect of *Krt16* on GSH synthesis as well as sex-specific fluctuations in redox balance. The latter may be explained by hormonal changes, such as the onset of puberty and the first LH surge, which respectively occur at 4 and 5.5 weeks in C57BL/6 female mice (Mayer et al., 2010); however, confirmation of this intriguing possibility would require a comprehensive analysis that is beyond the scope of this study.

Given that estrogen is a significant regulator of both redox balance and NRF2 signaling, we hypothesized that hormonal differences may also be responsible for the lack of response to the NRF2 inducer SF in *Krt16*$^{-/-}$ females. Estrogen receptor (ER)-α and ER-β have been

shown to directly inhibit (Yao et al., 2010) and activate (Sripathy et al., 2008) NRF2-mediated transcription, respectively, and accordingly differences in their regulation could account for the sex difference observed in the response of *Krt16*$^{-/-}$ footpad skin to SF. We compared the expression of ER-α and ER-β in *WT* and *Krt16*$^{-/-}$ female footpad skin, aged 3 to 8 weeks, to examine this possibility. When normalized to prepubescent levels (3 weeks old mice), the *ER-α* mRNA increased by >60 fold at 6 weeks of age in *WT* female paw skin. A similar increase in *ER-α* mRNA occurred in *Krt16*$^{-/-}$ females as well but took place slightly earlier, at 5.5 weeks of age (Figure 3A). *ER-α* mRNA levels decreased to pre-pubescent levels by 8 weeks of age in both genotypes. By comparison, the *ER-β* mRNA showed a more modest surge (12-14 fold increase) at 5.5 weeks of age in both *WT* and *Krt16*$^{-/-}$ female paw skin; again, this increase was transient (Figure 3B). The RNA findings were confirmed with indirect immunofluorescence (Figure 3C and 3D). Taken together with the GSSG and GSH/GSSG ratio data, these findings indicate that the development of the PPK phenotype in the paw skin of 5.5 weeks old *Krt16*$^{-/-}$ females (Figure 2C) coincides with both increased oxidative stress and changes in ER expression, the latter of which may be interfering with the SF-based therapeutic intervention. We hypothesized that the dominance of ER-α in *Krt16*$^{-/-}$ paw skin may block NRF2 activation despite treatment with SF. The potential connection between ER expression and NRF2 activation is further supported by our analysis of NRF2 status in *WT* male and female paw skin. Signal for both NRF2 and NRF2-P was detected in the epidermis of *WT* male and female paw skin at 5.5 weeks of age and in *WT* male paw skin at 6 weeks of age. However, NRF2-P is noticeably absent in the epidermis of 6-week-old *WT* female paw skin despite high levels of NRF2 being present (Supplemental Figure 4). Thus, a striking sex-specific lack of NRF2 activation occurs in *WT* female paw skin at a time when expression of ER-α is much greater than that of ER-β (Figure 3).

Other studies utilizing cell culture models revealed a synergism between sulforaphane and modulators of estrogen receptor signaling including 17β-Estradiol and exemestane, an aromatase inhibitor, although the underlying mechanism(s)remain(s) undefined (Angeloni et al., 2017, Liu and Talalay, 2013). Since ER-β reportedly exerts a positive impact on NRF2 signaling, we performed topical treatments of SF in combination with pre-treatment intra-peritoneal injections of diarylpropionitrile (DPN), an ER-β selective agonist that has been shown to also oppose ER-α activity (Song and Pan, 2012). Compared with vehicle-treated controls, *Krt16*$^{-/-}$ females treated with the dual treatment regimen showed a remarkable reduction in PPK lesion development (Figure 4A), with a significant decrease in PPK index from 33.7 ± 4.0 for SF + PBS treated to 0.5 ± 0.1 for SF + DPN treated *Krt16*$^{-/-}$ female mice (mean ± SEM; Student's t test, P value < 0.005; Figure 4B). Addition of DPN to the treatment regimen results in the normalization of epidermal thickness (Figure 4C) and restores the ability of SF to activate NRF2, as demonstrated by increased steady state levels of NRF2-P and NQO-1 in *Krt16*$^{-/-}$ female paw skin when assessed by both western blotting and indirect immunofluorescence (Figure 4D-E, quantification in Supplemental Figure 3). The dual treatment also has a beneficial impact on redox balance in *Krt16*$^{-/-}$ female paw tissue, given a 1.6 fold increase in GSH, a 2.7 fold decrease in GSSG, and a resulting GSH/GSSG ratio of 137.1 ± 7.8 for dual treatment compared to 38.1 ± 8.10 for SF alone (Figure 4F-H, Supplemental Table 4; Mean ± SEM). Treatment with DPN alone did not impact any

of these readouts (data not shown), suggesting that a synergistic effect of both DPN and SF is necessary and sufficient for the prevention of PPK and the improvement of redox balance of *Krt16*$^{-/-}$ female paw skin. We note that since the majority of *Krt16*$^{-/-}$ females exhibit a delay in lesion onset until 5.5. weeks of age, we were unable to accurately ascertain whether treatment with SF alone had a beneficial effect from 4 to 5.5 weeks of age, the time period during which ER-α levels are similar to prepubescent levels.

The following three possibilities could explain the beneficial impact of the addition of DPN to the SF treatment regimen of the *Krt16*$^{-/-}$ females. Firstly, the transient rise in ER-α mRNA and protein expression observed in *Krt16*$^{-/-}$ female paw skin suggests the possibility of a temporary shift of estrogen signaling towards ER-α signaling, which may have directly inhibited the ability of SF to activate NRF2. As an ER-α antagonist (Yao et al., 2010), DPN may have prevented the ER-α mediated blockade of SF's action. Secondly, the continual stimulation of ER-β secondary to DPN administration may have also contributed to the activation of NRF2 by SF. Either of these possibilities, and certainly a combination of both, would aid in skewing ER signaling away from ER-α, a negative regulator of NRF2 signaling (Yao et al., 2010), to ER-β, which favors NRF2 signaling (Sripathy et al., 2008). A third possibility is a synergistic effect of DPN with SF that is independent of estrogen signaling (e.g. anti-inflammatory and antioxidant effects) similar to that proposed for SF and exemestane, an aromatase inhibitor (Lui and Talalay, 2013).

The findings reported here point to significant differences in the mechanisms regulating NRF2 activity in keratinocytes of male and female epidermis and sexual dimorphism in the response to an NRF2 inducer in a model of PC-associated PPK. Whether and how this sex difference is manifested in individuals with PC and related disorders, or might impact response to therapy, is unknown at present and demands its own follow-up investigation. Interestingly, oral retinoid treatment has been shown to be less effective in female PC patients relative to males (Gruber et al., 2012). Further, our results significantly extend the case in support of K16 (Kerns et al., 2016) and estrogen receptor β (Song and Pan, 2012) as positive regulators of NRF2 function and the associated redox balance in skin keratinocytes.

Methods

Mouse studies, antibodies, biochemical and morphological analyses

These elements have been previously described (Hobbs et al., 2015, Kerns et al., 2016, Lessard and Coulombe, 2012) and are detailed under the “Supplemental Data” section available online.

Preparation of SF and DPN

Stock solutions of sulforaphane (SF), obtained from LKT Laboratories Inc., were prepared at 0.1 M in DMSO vehicle and diluted to the desired final concentration in jojoba oil (MP Biomedicals LLC) on the day of the treatment as described (Dinkova-Kostova et al., 2007, Kerns et al., 2010). Stock solutions of DPN (Sigma-Aldrich) were prepared at 1.0 μM in DMSO and diluted to the desired final concentration for intra peritoneal (i.p) injection on the day of the treatment (Campbell et al., 2010).

Human studies

Punch biopsies (3 mm) were obtained from individuals with PC with informed written consent as described (Cao et al., 2015), and analyzed for protein expression by immunohistochemistry. Cryosections (50 μm) were cut and incubated with primary antibodies and Alexa Fluor-conjugated secondary antibodies for indirect immunofluorescence (Kerns et al., 2016).

Study approval

All studies involving mice were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Human plantar skin biopsies were obtained using standard surgical techniques with patient consent under W-IRB #2004/0468/1057496.

Image quantification and Statistics

Immunofluorescence micrographs were quantified using ImageJ. Unpaired 2-tailed Student's *t* test were performed when appropriate. Significance was set at P value less than 0.05. Quantification data were presented in mean ± SEM. For comparison of the onset of PPK onset between sexes, log rank (Mantel-Cox) test was performed and relative risk was calculated for each time point assessed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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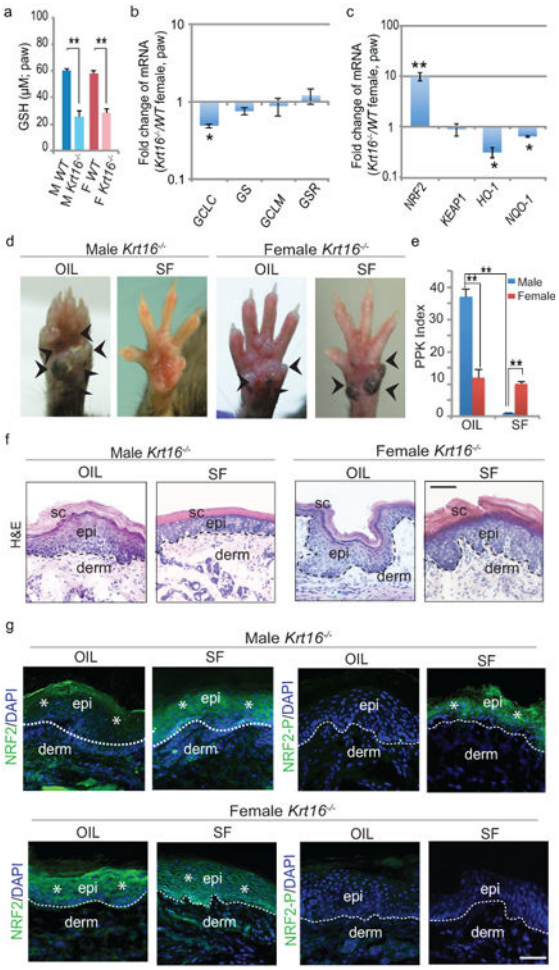

Figure 1.

Sex-specific outcomes following topical SF treatment of paw skin in *Krt16*$^{-/-}$ mice. (a) GSH for male and female *WT* and *Krt16*$^{-/-}$ paw skin. (b,c) Relative fold change in mRNA levels of GSH related enzymes (b) and NRF2 signaling pathway related targets (c). For a-c, mice are 4 weeks of age, data represents mean ± SEM of 3 biological replicates, Student's t test, *P value < 0.05, **P value < 0.01. (d) Representative images of male and female *Krt16*$^{-/-}$ paws following SF or vehicle (OIL) treatment. Arrowheads point to affected areas. (e) PPK index following SF or vehicle (OIL) treatment per sex. Mean ± SEM. n = 10 mice per group. (f) H&E stained histological sections of male and female *Krt16*$^{-/-}$ paw skin following SF or vehicle (OIL) treatment. Scale bar, 50 μm. (g) Indirect immunofluorescence for NRF2 and NRF2-P in OIL- and SF-treated male and female *Krt16*$^{-/-}$ paw skin. DAPI, nuclear staining; Sc, stratum corneum; epi, epidermis; derm, dermis. Dotted lines mark the dermo-epidermal junction and asterisks mark areas of increased intensity in g. Scale bar, 50 μm. For d-g, mice are 8 weeks of age.

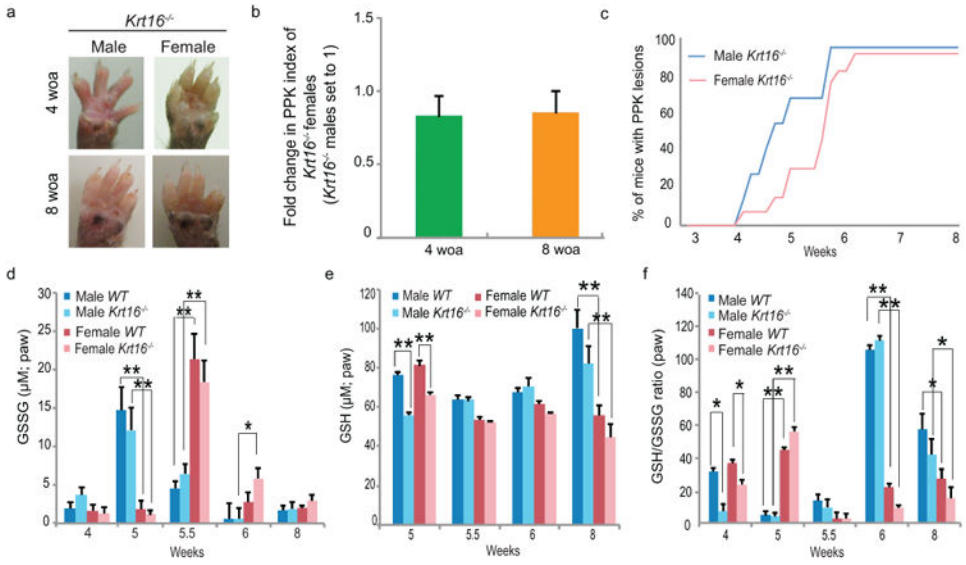

Figure 2.

Comparison of male and female *Krt16*$^{-/-}$. (a) Macroscopic images of paws from male and female *Krt16*$^{-/-}$ mice at 4 and 8 weeks of age (woa). (b) Fold change in PPK index of *Krt16*$^{-/-}$ females at 4 and 8 weeks of age relative to age matched male *Krt16*$^{-/-}$ males. Data represents mean ± SEM. (c) Onset of PPK lesions in *Krt16*$^{-/-}$ mice per sex. n = 12 per group. (d-f) GSSG (d), GSH (e), and GSH to GSSG ratio (f) for male and female *WT* and *Krt16*$^{-/-}$ paw skin. For e, GSH of 4 week old paw skin is shown in Figure 1. For e-f, data represents mean ± SEM of 3 biological replicates, Student's t test, *P value < 0.05, **P value < 0.01.

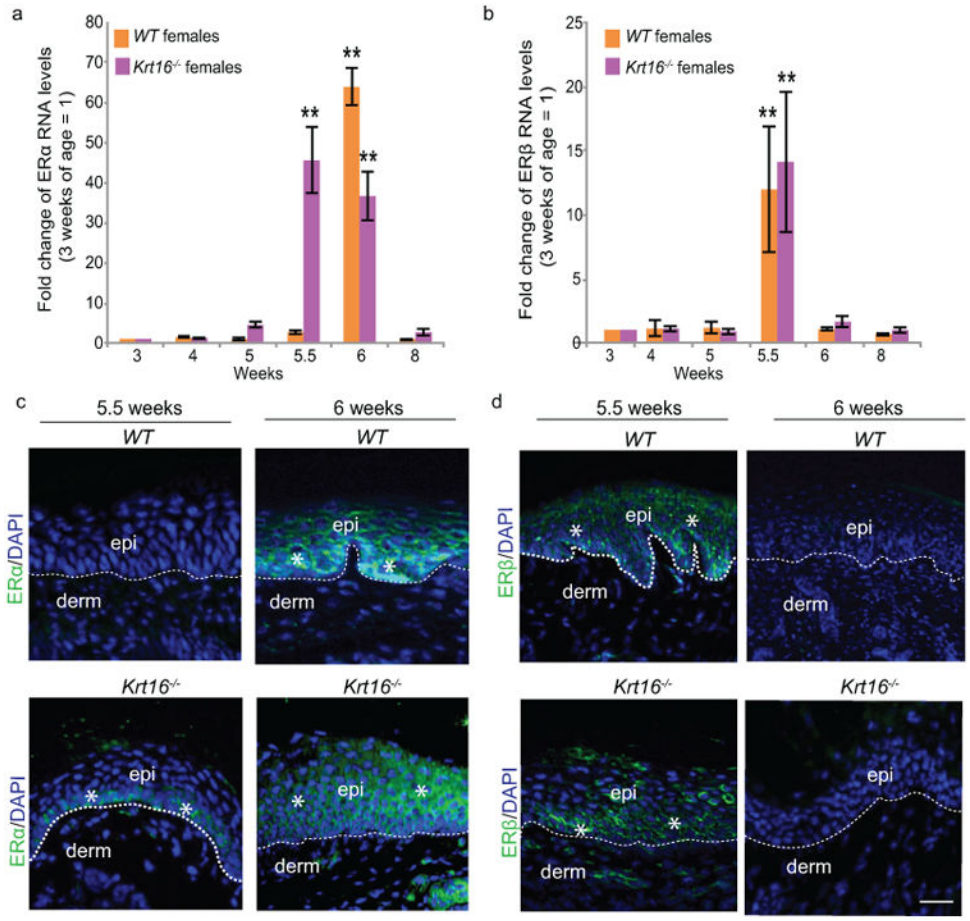

Figure 3.

ERα and ERβ expression in female *WT* and *Krt16*$^{-/-}$ murine paw skin. Relative fold change in mRNA levels of *ERα* (a) and *ERβ* (b). For A-B, data represents mean ± SEM of 3 biological replicates, Student's t test, *P value < 0.05, **P value < 0.01.Indirect immunofluorescence for ERα (c) and ERβ (d) for *WT* and *Krt16*$^{-/-}$ female murine paw skin at 5.5 and 6 weeks of age. epi = epidermis; derm = dermis. Dotted line marks the dermo-epidermal junction. Scale bar = 50 μm. Asterisks mark areas of increased immunofluorescence signal.

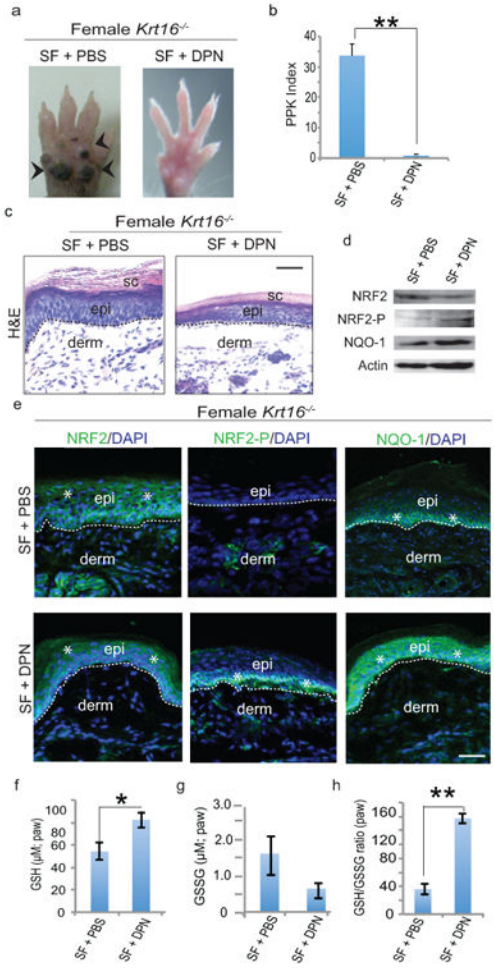

Figure 4.

Dual treatment with SF and ERβ agonist DPN prevents the development of PPK lesions in *Krt16*$^{-/-}$ females. (a-b) Representative images (a), quantitation of PPK index (b), and H&E staining (c) of *Krt16*$^{-/-}$ female paws treated with SF+PBS (vehicle for DPN) or SF+DPN. Scale bar, 50 μm. For each treatment group, n = 6. (d-e) Representative western blots (d) and immunofluorescence staining (e) for NRF2, NRF2-P, and NQO-1. For d, actin is a loading control. For e, DAPI, nuclear staining; epi, epidermis; derm, dermis. Dotted lines mark the dermo-epidermal junction and asterisks mark areas of increased intensity in Scale bar, 50 μm. Measurements of GSH (f), GSSG (g), and GSH/GSSG (h) for female *Krt16*$^{-/-}$ paws treated with SF + PBS or SF + DPN. For f-h, data represents mean ± SEM of 6 biological replicates per group. For b, f-h, Student's t test, *P value < 0.05, **P value < 0.01.