Targeted disruption of glutathione peroxidase 4 (GPx4) in mouse skin epithelial cells impairs postnatal hair follicle morphogenesis that is partially rescued through inhibition of COX-2

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Abstract

Selenoproteins are essential molecules for the mammalian antioxidant network. We previously demonstrated that targeted loss of all selenoproteins in mouse epidermis disrupted skin and hair development and caused premature death. In the current study we targeted specific selenoproteins for epidermal deletion to determine whether similar phenotypes developed. Keratinocyte-specific knockout mice lacking either the glutathione peroxidase 4 (GPx4) or thioredoxin reductase 1 (TR1) gene were generated by cre-lox technology using K14-cre. TR1 knockout mice had a normal phenotype in resting skin while GPx4 loss in epidermis caused epidermal hyperplasia, dermal inflammatory infiltrate, dysmorphic hair follicles and alopecia in perinatal mice. Unlike epidermal ablation of all selenoproteins, mice ablated for GPx4 recovered after 5 weeks and had a normal lifespan. GPx1 and TR1 were upregulated in the skin and keratinocytes of GPx4 knockout mice. GPx4 deletion reduces keratinocyte adhesion in culture and increases lipid peroxidation and COX-2 levels in cultured keratinocytes and whole skin. Feeding a COX-2 inhibitor to nursing mothers partially prevents development of the abnormal skin phenotype in knockout pups. These data link the activity of cutaneous GPx4 to the regulation of COX-2 and hair follicle morphogenesis.

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morphogenesis and provide insight into the function of individual selenoprotein activity in maintaining cutaneous homeostasis.

Introduction

Selenoproteins are important antioxidant enzymes in mammals, harboring the 21st amino acid, selenocysteine (Sec), in their catalytic domain (Kryukov et al., 2003). As observed for all selenoproteins (Kumaraswamy et al., 2003 and references therein), knockout mouse models for individual selenoproteins like glutathione peroxidase 4 (GPx4) (Imai et al., 2003; Yant et al., 2003); thioredoxin reductase 1 (TR1) (Jakupoglu et al., 2005; Bondareva et al., 2007) and thioredoxin reductase 2 (TR2) (Conrad et al., 2004), are also embryonic lethal, establishing the importance of glutathione peroxidases and thioredoxin reductases in development.

Glutathione peroxidases react with $\text{H}_2\text{O}_2$ and fatty acid hydroperoxides (Brigelius-Flohé and Kipp, 2009), and among its family members, five are selenoproteins in human and four in mice (Kryukov et al., 2003). GPx4, or phospholipid hydroperoxide glutathione peroxidase (PHGPx), is the only member of the family directly reducing phospholipid hydroperoxides in membranes and lipoproteins at the expense of glutathione (Ursini et al., 1982; Thomas et al., 1990a) or protein-thiol groups (Conrad et al., 2005; Mauri et al., 2003). GPx4 functions as a redox sensor to induce cell death (Seiler et al., 2008), protects against lipid hydroperoxide damage in neurodegenerative diseases (Yoo et al., 2010; Wirth et al., 2010) and is regulated in cancer (Liu et al., 2006; Cejas et al., 2007). GPx4 overexpression in cells renders them more resistant to oxidative stress causing agents (Arai et al., 1999) and inhibits the enzymatic activity of many lipoxygenases and cyclooxygenases (Chen et al., 2003; Huang et al., 1999). GPx4 knockout embryos have disorganized germ layers lacking differentiation into structures (Imai et al., 2003; Yant et al., 2003), while cell lines generated from GPx4 null mouse embryos are susceptible to inducers of oxidative stress (Yant et al., 2003), confirming the importance of GPx4 in the antioxidant network.

Thioredoxin reductases in combination with thioredoxins, constitute an important oxidoreductase system in mammals (Nordberg and Arner, 2001), having cytosolic (TR1) (Gladyshev et al., 1996), mitochondrial (TR2) (Gasdaska et al., 1999), and testis-specific isoforms (Sun et al., 2001). The catalytically active penultimate Sec residue at the C-terminal domain gives uniqueness to their function (Gladyshev et al., 1996; Mustacich and Powis, 2000). TR1 is the most studied member of this group, owing to roles in redox regulation, cell proliferation, DNA repair, angiogenesis, cell signaling and antagonistic roles in preventing and promoting cancer (Hatfield et al., 2009). Knockout mouse models established the importance of TR1 in development, revealing embryonic lethality along with severe growth retardation and defective cell proliferation (Jakupoglu et al., 2005).

Antioxidant enzymes are essential to neutralize the damaging effects of reactive oxygen species (ROS) in mammalian skin generated through atmospheric oxygen, environmental toxins, pollutants and UV light, which may initiate several skin disorders including malignancies (Guyton and Kensler, 1993; Richelle et al., 2006). We recently demonstrated
the importance of selenoproteins as antioxidants in skin, through their targeted removal in mouse epidermis, which generated mice with stunted growth, gross abnormalities of skin, hair loss and premature death, in addition to ROS accumulation and lipid peroxidation (Sengupta et al., 2010). This study also revealed GPx4 and TR1 as two of the most abundantly expressed selenoproteins in the epidermis of skin and cultured keratinocytes, corroborating earlier reports of functional significance of glutathione peroxidase and thioredoxin reductase protein families in skin (Richelle et al., 2006; Sohn et al., 2007). In light of these observations and earlier reports showing that knockout mouse models of GPx4 or TR1 are embryonic lethal, the present study was undertaken to examine whether one of these selenoproteins may be responsible, at least in part, for the many phenotypic and histological changes resulting from the ablation of selenoproteins in skin (Sengupta et al., 2010). Though no obvious phenotypic changes were observed for targeted removal of TR1 in skin, the lack of GPx4 modulated postnatal hair follicle morphogenesis, inflammatory cell infiltration and epidermal proliferation, associated with elevated expression of COX-2. With time, the phenotype resolved in association with compensatory upregulation of other selenoproteins including TR1 and GPx1.

Results

Deletion of GPx4 in mouse keratinocytes alters hair follicle morphogenesis

We generated two mouse models, with specific deletion of GPx4 or TR1 in K14 expressing cells of skin. Targeted removal of TR1 did not cause any apparent alteration in skin phenotype, life-span or weight of knockout mice, with both male and female knockouts being fertile (data not shown). Ablation of GPx4 in skin keratinocytes generated knockout pups, born with the expected Mendelian ratio (24.49%; Supplementary Figure S1a online), but exhibited visible skin abnormalities during the second week after birth. For all experimental purposes, knockout pups (K14-cre; GPx4fl/fl) were compared to control littermates (K14-cre; GPx4fl/+ or GPx4fl/fl) (Supplementary Figure S1b online). The extent of recombination of GPx4 in several tissues was determined in knockout and control (K14-cre; GPx4fl/fl) littermates, where the ΔGPx4 allele was detected in skin of both genotypes and tongue of knockout mice (Supplementary Figure S1c online). Partial recombination in the skin of control heterozygous animals resulted due to the presence of K14-cre, and absence of ΔGPx4 allele in any other tissue established specificity of recombination. Subsequent breeding showed that both male and female GPx4 knockout mice were fertile.

Ablation of GPx4 did not lead to neonatal death but monitoring pups for 60 days post-birth showed that ~80% knockout mice survived to adulthood post-weaning (Figure 1a). The body weight of knockout pups was similar to control pups for about 1 week following birth, beyond which their weight gain was slower and a difference in body weight remained throughout the time observed (Figure 1b).

Skin of knockout mice was normal at birth (Supplementary Figure S2 online), but around 1 week of age, when control mice developed visible hair, the knockouts were still hairless over most of their bodies (Figure 1c) independent of skin color (Supplementary Figure S1d online). Over the next several weeks, when control pups had developed a uniform hair coat, hair growth on knockout mice was sparse, with areas of focal alopecia. This variation in coat...
quality continued as the mice aged. Histologically, knockout epidermis was hyperplastic (arrow head), and hair follicles were misaligned with marked outer root sheath (ORS) hyperplasia and abnormally shaped hair bulbs with little evidence of hair formation during the first hair cycle (Figure 2). The stratum corneum was thickened in young knockout mice (asterisk) along with a dense cellular dermis and decreased subcutaneous fat (triangle). While the hair cycle was in telogen in control mice at 3 weeks as expected, knockout mice had begun a second anagen. Accelerated entry into anagen was also observed in knockout mice in the next hair cycle at 49 days, with control follicles still in telogen. Nevertheless, by 28 days much of the alterations in histology had resolved in knockout skin, although visible hair remained sparse and sebaceous glands were enlarged. In contrast, hair follicle density was reduced in comparison to controls only during the perinatal period (Supplementary Figure S1e online), whereas hair follicle density was equivalent between knockout and control skin in older mice, suggesting the reduction in GPx4 impaired hair maturation. Histology of skin from newborn pups or tongue and whiskers from adult littermates did not reveal any significant change between the control and knockout pups (Supplementary Figure S2 online).

**Lack of GPx4 alters keratinocyte proliferation, differentiation markers and induces infiltration of inflammatory cells**

Using specific antibodies, we documented biochemical changes in perinatal GPx4 knockout skin epithelium *in situ* associated with the altered cutaneous morphology. Unlike compartmentalized proliferation (Ki67) in basal epidermis and hair follicle bulbs in control skin, proliferating epithelial cells were abundant throughout the skin of knockout mice, particularly in the ORS of follicles (Figure 3) and basal epidermal cells. Likewise, there was a marked expansion of keratin 14 (K14) positive cells and aberrant expression of keratin 6 (K6) in the epidermis and outer root sheath. Further, the epidermal terminal differentiation markers keratin 1 (K1) and loricrin were detected in hair follicle structures, suggesting an abnormal “epidermal like” differentiation in the absence of GPx4. Of interest is the “normalization” of these differentiation markers in knockout skin as a function of age, associated with the normalization of the morphological changes (Supplementary Figure S3 online). Using similar *in situ* staining, we explored the composition of the extensive dermal cellularity to determine whether these were inflammatory cells, as observed earlier with complete ablation of selenoproteins in skin epithelium (Sengupta et al., 2010). Supplementary Figure S4 online confirms the extensive infiltration of macrophages and granulocytes within the first 10 days of life for knockout pups and the gradual resolution of inflammation as morphological changes in skin resolve with aging.

**Deletion of GPx4 causes cell autonomous changes in isolated keratinocytes**

To rule out a systemic contribution to the altered skin phenotype and confirm the cell autonomous defect of GPx4 ablation in keratinocytes, we grafted freshly isolated primary keratinocytes and their corresponding dermal cells from each genotype onto the dorsum of nude mice. Grafts of control cells produced normal haired skin after 3 weeks, while knockout grafts were contracted with sparse hair growth (Figure 4a). Isolated cultured primary keratinocytes from knockout mice grew poorly on plastic culture dishes (Figure 4b, c), showing a 37% reduction in cell number in comparison to those from control mice. This
could be corrected by coating plates with a mixture of collagen I and fibronectin (ColI/FN), where the knockout keratinocytes grew as well as control keratinocytes (Figure 4d, e). Hence, in subsequent experiments, cells were grown on such coated plates. The number of GPx4 keratinocytes that adhered to plastic over time was compromised but that improved when the plates were coated with ColI/FN (Figure 4f).

Cultured keratinocytes were radiolabeled with selenium $^{75}$Se to visualize the expression of selenoproteins upon GPx4 ablation. Though intensities of most selenoproteins remained unchanged, GPx1 and TR1 were elevated in cultures from knockout mice relative to control keratinocytes (Figure 4g). This observation was corroborated through immunodetection of GPx1 and TR1 in lysates from cultured keratinocytes (Figure 4h). Immunodetection and selenium $^{75}$Se-labeling also revealed a faint band corresponding to GPx4 in keratinocyte cultures. This could result from contaminating cells other than keratinocytes (e.g. melanocytes, fibroblasts) that are known to be present in these cultures (Sengupta et al., 2010) or from incomplete recombination in the crossbreeding. The latter seems less likely as GPx4 immunostaining was not detected in epidermis or hair follicles from knockout mice (Figure 4i). In contrast, enhanced staining of TR1 was detected in knockout epidermis and hair follicles, consistent with the enhanced expression detected in isolated keratinocytes. Unfortunately, antibodies capable of detecting GPx1 in skin in vivo are not available, nonetheless, quantitative real-time PCR (q-PCR) revealed elevated levels of GPx1 and TR1 transcripts in cultured keratinocytes from knockout mice (Figure 4j).

**COX-2 expression and lipid peroxidation increase in keratinocytes lacking GPx4**

Cultured keratinocytes were examined for gene expression profile associated with targeted removal of GPx4 (GEO accession number GSE34215) and the top 25 genes displaying ≥2-fold variation (increase or decrease) and p value ≤0.05 (Supplementary Table S1 online) are depicted along with some major functional classes influenced by the loss of GPx4 (Supplementary Figure S5a online). Several candidate genes from the regulated functional pathways were examined through q-PCR (Supplementary Figure S5b online) and results corroborate changes observed in microarrays for most genes. Networks were generated for differentially regulated genes through Ingenuity Pathway Analysis (IPA), to explore pathway(s) and identify target gene(s), which were substantially affected by the loss of GPx4 in keratinocytes (Supplementary Figure S6 online). One key gene regulated through loss of GPx4 is PTGS2 (prostaglandin-endoperoxide synthase 2), or COX-2 (cyclooxygenase-2). Cyclooxygenases are proteins, whose enzymatic activity is inhibited by GPx4 (Imai and Nakagawa, 2003; Huang et al., 1999); hence, we examined the levels of COX-2 in GPx4 knockout cells and observed an increase in COX-2 mRNA (Figure 5a, left panel), concurrent with elevated protein levels (Figure 5a, right panel) in cultured keratinocytes from knockout mice. Elevated COX-2 levels were also associated with increased levels of PGE2 in skin of knockout pups (Figure 5b). GPx4 protects cells by reducing membrane lipid hydroperoxides, which are key activators of lipoxygenase and cyclooxygenase. Hence, we explored whether the loss of GPx4 influenced lipid peroxidation by examining levels of 4-hydroxynonenal (4-HNE), a marker of lipid peroxidation, and observed ~1.6 fold increase in keratinocytes derived from knockout mice in comparison to their control counterpart (Figure 5c). In vivo, COX-2 is detected in a few hair follicle cells of
normal mice (Figure 5d). In contrast, COX-2 positive cells are relatively abundant in skin from knockout mice, where they are localized particularly in the prominent K14 positive expanded follicle epithelium and the ORS of more organized follicles at day 16 (Figure 5d). Total skin from control and knockout littermates were examined for selected selenoproteins and genes involved in inflammatory response by q-PCR. A significant increase in transcripts for inflammatory response related genes (Cxcl1, Il1b, S100a8, Tnfa, Ptgs2) along with an increase in TR1 and to a lesser extent GPx1 transcripts, similar to cultured keratinocytes, were detected (Figure 5e). The connection of selenoprotein deficiency and COX-2 upregulation is seen most prominently in mice lacking all selenoproteins in the skin epithelium (Supplementary Figure S7 online). Under these conditions COX-2 is highly upregulated in both epidermis and abnormal hair follicles. To determine the contribution of COX-2 to the hair and skin phenotype, we added a COX-2 inhibitor, celecoxib, to the diet of nursing mothers giving rise to control and knockout pups and examined the phenotype of the offspring (Figure 5f). Control pups were not affected by exposure to celecoxib in milk, but the skin phenotype of knockout littermates was substantially improved by 17 days (Figure 5f). Histological examination revealed a significant decrease in aberrant follicles, with skin histology of knockouts resembling those of control pups (Figure 5g). When we examined the skin of control and knockout pups from litters where the nursing mother was treated with a celecoxib diet (Figure 5h), there was a reduction in the elevated transcripts for Cxcl1 and S100a8, suggesting that at least part of the inflammatory response is mediated by the increased activity of COX2. These results support an active contribution of COX-2 and prostaglandins to the GPx4 depleted skin phenotype.

**Discussion**

GPx4 and TR1 are important regulators of cellular ROS (Nordberg and Arner, 2001; Hatfield et al., 2006), with their ablation being embryonic lethal in mice (Imai et al., 2003; Yant et al., 2003; Jakupoglu et al., 2005). Hence, conditional knockout mouse models were generated to elucidate their functions in various organs and tissues (Conrad, 2009). However, skin was not examined in previous targeted deletion studies and in light of their critical roles as antioxidants and the severe developmental outcome due to lack of all selenoproteins in skin epithelia (Sengupta et al., 2010), this study was undertaken to evaluate the requirement for either GPx4 or TR1 as individual selenoproteins known to be expressed prominently in skin.

Targeted removal of TR1 in keratinocytes did not cause apparent changes in skin phenotype or host growth, suggesting that TR1 may not be critical for skin function in the resting state or its absence was compensated by other proteins. However, additional studies on these mice will be required to determine whether keratinocyte TR1 function contributes to cutaneous homeostasis under conditions of stress. In contrast, lack of GPx4 in keratinocytes caused a major alteration in hair follicle morphogenesis, focal alopecia, epidermal hyperplasia and a marked dermal inflammatory infiltrate of neutrophils and macrophages. This phenotype is virtually identical to that described for complete knockout of selenoproteins reported previously with the same K14 targeting vector (Sengupta et al., 2010). This suggests that GPx4 is a major regulator of the oxidant environment required for the normal development and maturation of hair follicles in the first hair cycle. In contrast to the fatal outcome from
loss of all cutaneous epithelial selenoproteins, individual loss of GPx4 can be compensated by elevation of GPx1 and TR1 and possibly others that were not examined. These compensations were not observed in mouse fibroblasts lacking GPx4 (Yoo et al., 2010), suggesting overlapping functional similarity of GPx1 and TR1 with GPx4 in keratinocytes. However, GPx1/GPx2 double knockout mice do not have a similar skin phenotype (Esworthy et al., 2001) further suggesting that GPx4 has a major anti-oxidant function in the skin. Of interest is the striking development of colitis in the GPx1/GPx2 double knockout mice indicating organ specific function for the GPx family of selenoproteins. GPx4 is important during early stages of development, as germ layers in embryos from GPx4 knockout mice lack differentiation into structures (Imai et al., 2003; Yant et al., 2003). Reduction in cell attachment to non-physiological substrates and elevated membrane lipid peroxides could contribute to this.

While reversal of extensive alopecia occurs after several weeks of life, a sparse hair coat persists even though hair follicle density is restored. This could be the result of telogen follicles failing to reenter the hair cycle. The physiological basis for the failure is unclear but could be related to the extensive inflammatory infiltrate that occurs around damaged hair follicles and the follicular hyperkeratosis in the first hair cycle. Post inflammatory alopecia is a well known skin disorder in humans and the consequence of many underlying inflammatory diatheses (Harries and Paus, 2010), with the loss of follicle stem cells as a result of inflammation being a contributing factor (Zhou et al., 2012).

We employed a multi-technique approach to understand the influence of GPx4 ablation on other genes in keratinocytes and identified COX-2 as a potential candidate, which was elevated in keratinocyte cultures and skin epithelium from knockout mice. Previous studies have linked downregulation of selenoproteins (GPx2) in intestinal cells or (DIO2 in chondrocytes with upregulation of COX-2 (Brigelius-Flohe and Kipp, 2012; Cheng et al., 2012). An important enzyme in the prostaglandin synthesis pathway, COX-2, is almost undetectable in skin under physiological conditions, but is induced in response to stimuli (Müller-Decker and Fürstenberger, 2007). It is noteworthy that transgenic mice over-expressing COX-2 in keratinocytes show delayed morphogenesis of pelage hair follicles, aberrant hair follicles, hyperplasia of tail epidermis and lifelong alopecia (Neufang et al., 2001; Bol et al., 2002), features also observed in the current study. However, mice over-expressing COX-2 did not exhibit inflammation. GPx4 protects cells by reducing hydroperoxides, which activate lipoxygenase and cyclooxygenase, causing inflammation, apoptosis and altered cellular signaling (Brigelius-Flohe and Kipp, 2009; Imai and Nakagawa 2003; Conrad et al., 2010). Membrane-bound hydroperoxides can only be reduced by GPx4 and its absence leads to accumulation of hydroperoxides, noted through increased 4-HNE in GPx4 depleted keratinocytes, highlighting the role of GPx4 in repairing membrane lipid hydroperoxides in skin (Thomas et al., 1990b; Sattler et al., 1994). Elevated 4-HNE levels can induce COX-2 expression in mammalian cells by stabilizing its mRNA (Kumagai et al., 2000; Kumagai et al., 2004). In light of current observations, we presume that loss of GPx4 in keratinocytes leads to increased lipid peroxidation, accumulation of hydroperoxides and its by-products like 4-HNE, which in turn could elevate COX-2 and prostaglandins in the skin (Supplementary Figure S8 online). It has been reported previously.
that topical application of lipid peroxides causes apoptosis of the hair follicle matrix cells (Naito et al., 2008). The mixed phenotype of inflammation and alopecia we observed is likely to involve apoptosis and direct necrotic damage to hair follicles as a result of lipid peroxides and pro-inflammatory cytokines and chemokines, both elevated in GPx4 depleted epidermis and keratinocytes. Undoubtedly, elevated COX2 and prostaglandins contribute to the hair follicle phenotype as demonstrated by the partial resolution of hair follicle abnormalities, resumption of hair growth and reduction of some inflammatory cytokines in knockout pups exposed to celecoxib through the nursing mother (Figure 5g, h). It should be noted that COX-2 may not be the only molecule contributing to the phenotype, but seems to be a key player in the process. Interestingly, even more intense COX-2 expression was detected in skin sections from mice with complete lack of selenoproteins in keratinocytes, suggesting that the presence of some selenoproteins in skin epithelium can moderate COX-2 levels. Since dietary selenium can modulate the levels of selenoproteins in vivo and selenium is known to protect keratinocytes from oxidizing events such as UVB radiation (Rafferty et al., 1998), illuminating the functions of individual selenoproteins in skin keratinocytes may reveal fundamental principles of environmental protection provided by the most important organ that interfaces with the outside world.

Materials and Methods

Generation of knockout mice

Mice floxed for GPx4 (GPx4fl/fl; Seiler et al., 2008) or TR1 (TR1fl/fl; Jakupoglu et al., 2005) were crossed with K14-cre transgenic mice to generate fertile heterozygous offspring bearing K14-cre. The resultant heterozygous male offspring bearing K14-cre (K14-cre; GPx4fl/+ or K14-cre; TR1fl/+ ) were crossed with floxed females to obtain knockout pups.

Histological and immunochemical analysis

Littermates were sacrificed at various ages by CO2 inhalation and samples from identical regions of back skin were processed for molecular, histological and histopathological examinations as described (Sengupta, et al., 2010). Paraffin-embedded tissue sections were used for immunohistochemistry by deparaffinizing in xylene, followed by alcohol rehydration. After quenching endogenous peroxidases, slides were rinsed in PBS, and when required, an antigen retrieval step was carried out for 10 min in preheated citrate buffer (pH 6.0). Slides were subsequently incubated overnight with the required primary antibody at 4°C (with the exception of COX-2 antibodies, which was done at room temperature), followed by incubation with biotin-conjugated secondary antibody. Vectastain Elite ABC kit and DAB were used for detection, following manufacturer’s instructions and slides were counterstained with hematoxylin. Appropriate pre-immune sera controls were used to rule out nonspecific immunostaining of tissue sections; however, some nonspecific staining was seen in sebaceous glands.

Primary keratinocyte culture, adhesion assay and molecular analysis

Primary keratinocytes were isolated and cultured from newborn mice as described, with all media; both for preparation and culture of primary keratinocytes, being supplemented with 100 nM sodium selenite (Sengupta et al., 2010). In some experiments, culture plates pre-
coated with attachment factors collagen IV, Matrigel, or a mixture of collagen I and fibronectin (ColI/FN) were used for analysis (Sengupta et al., 2010; Lichti et al., 2008). Equal number of Mouse Equivalent (ME) cells from control and knockout mice was plated for experimental purposes and cultures were generally analyzed 2–3 days after plating. To determine the relative confluency following growth, images of cells from 4–5 fields of respective plates were captured, cells in each field counted using the ImageJ software and average count per field (under identical magnification) for each genotype was plotted as a bar-graph. Grafting of keratinocytes onto nude mice and cell adhesion studies in 12-well tissue culture plates were performed as described (Lichti et al., 2008; Sengupta et al., 2010).

**Labeling of selenoproteins in cultured keratinocytes**

Primary keratinocytes (5x10^5 cells/well) from control and knockout mice were seeded onto a 6-well plate and cultured for 3 days. Cells were washed once with PBS and labeled for 12 h with 50 μCi/ml of [75Se] in LoCa medium at pH 7.2. Following incubation, labeled cells were harvested and processed as described (Sengupta et al., 2010).

**Microarray and quantitative real-time PCR (q-PCR)**

Detailed protocol for microarray analysis and q-PCR are described in Supplementary Materials and Methods online.

**Diet study**

To study the effect of COX-2 selective inhibitor celecoxib on skin phenotype induced by knocking down GPx4 in keratinocytes, standard purified AIN-93G rodent diet without or with 500 ppm Celecoxib were made to order by Dyets, Inc. (Bethlehem, PA, USA). Inhibitor and control diet pellets were fed to separate nursing females starting on day 1 after giving birth. The pups were sacrificed between 17–19 days after birth for histological examination. In cases of large litters, littersmates were genotyped on day 1 after birth in order to allow identification of control pups with the aim of reducing their numbers, and thus reducing the competition during feeding.

**Prostaglandin assay**

The entire skin from control and knockout mice was quickly removed following sacrifice by CO₂ asphyxiation, and rapidly frozen in liquid nitrogen. Comparable regions of skin from control and knockout pups were analyzed for PGE₂ content as described earlier (Ansari et al., 2007).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Survival rate, growth curve and phenotype of GPx4 knockout and control mice
(a) Kaplan-Meier survival plot of GPx4 knockout (– – –, n=30) and control mice (——, n=54), with a cumulative survival proportion of 0.76 for knockout pups. (b) Growth curve of knockout (– – –, n=14) and control (——, n=23) littermates showing weight variations in the two mouse lines. (c) Phenotype of age matched knockout (KO) and control (CT) mice showing defective hair development, a less dense and furry hair coat in knockout offspring compared to control littermates. Error bars represent mean ± S.D. **, p ≤ 0.001; *, p ≤ 0.05.
Figure 2. Postnatal hair follicle morphogenesis and cycling in GPx4 knockout mice
Back skin sections from control and knockout mice at various ages were stained with H&E for histological examination. Sections from knockout mice show hair follicles of abnormal morphology that are misaligned and irregularly spaced. A thickened cornified layer (asterisk) above a hyperplastic epidermis (arrow head), dense cellular dermis and decreased number of fat cells (triangle) were observed in knockout mice until 3 weeks of age and then the phenotype resolves. Scale bar: 100 μm.
Figure 3. Altered expression of proliferation and keratinocyte differentiation markers in skin sections of mice lacking GPx4 in keratinocytes

Back skin sections from age matched control and knockout mice were stained for Ki67 to examine proliferation and for keratin-14, keratin-1, loricrin and keratin-6 to study differentiation. Increased Ki67 staining was detected in matrix cells of hair follicle bulbs, basal layer of epidermis and ORS of hair follicles of 10 day old knockout pups. Aberrant expression of markers of epidermal differentiation was noted in 12 day old knockout skin sections. Scale bar: 100 μm.
Figure 4. Keratinocytes lacking GPx4 have altered adhesion properties and modified selenoprotein expression

(a) Freshly isolated keratinocytes and corresponding dermal cells from control and knockout mice were grafted onto backs of nude mice and examined 21 days post grafting. Scale bar: 1 mm. (b, c) Keratinocytes from knockout pups attach poorly to plastic culture dishes and display elongated and refractile morphology. Scale bar: 50 μm. (c; n=5). (d, e) Keratinocytes from knockout pups attach to culture plates coated with ColI/FN identically as keratinocytes from control littermates and display cuboidal morphology. Scale bar: 50 μm. (e; n=5) (f) Adhesion differences for keratinocytes from control and knockout mice following 30, 60 and 120 min of attachment on uncoated and ColI/FN coated plates (n=3). (g) Expression of selenoproteins in keratinocytes labeled with [75Se]. Right panel shows incorporation of [75Se] into proteins and left panel, Coomassie blue staining. Identification of several selenoproteins is designated on the right of the figure. (h) Western blot for TR1, GPx1 and GPx4 in lysates of cultured keratinocytes, with tubulin as loading control. (i) Immunodetection of GPx4 (10 days old) and TR1 (12 days old) in back skin sections of
Sections from control mice showed GPx4 staining in outer root sheath, with no staining in knockout section. TR1 staining was mostly confined to the inner root sheath of control mice, while in knockouts, it was also observed in the hyperplastic epidermis. Enlarged areas of stained region in control sections are shown alongside. Scale bar: 100 μm. (j) q-PCR analysis showing relative fold change in GPx1 and TR1 in cultured keratinocytes from GPx4 knockout mice. CT and KO designate control and knockout mice respectively. Data represent the mean values ± S.D. **, $p \leq 0.001$; *, $p \leq 0.01$; NS, not significant.
Figure 5. COX-2 and other inflammatory mediators are elevated in keratinocytes and skin of GPx4 knockout pups and mediate the altered skin phenotype
(a) Left panel, q-PCR analysis of COX-2 expression in cultured keratinocytes from control and knockout littermates. \((n=3)\). Right panel, immunodetection of COX-2 in lysates of cultured keratinocytes, with tubulin as loading control. (b) PGE2 levels in skin from knockout pups relative to control littermates \((n=4)\). (c) ELISA assay for 4-hydroxynonenal (4-HNE) in protein lysates from cultured keratinocytes to assess lipid peroxidation \((n=3)\). (d) Immunodetection of COX-2 in back skin sections of age matched control and knockout mice. Faint staining was noted in hair bulbs of 12 d old control mice (arrowhead), while scattered staining was observed in ORS of misshapen hair bulbs (arrows) of various ages for knockout mice. Scale bar: 100 μm. (e) q-PCR analysis from skin biopsies of 15 day old control and knockout mice for selected selenoproteins and genes involved in inflammatory response \((n=4)\). (f) Representative phenotype of 17d old control and knockout pups nursed by mothers fed either control or celecoxib (COX-2 inhibitor) containing diets beginning 24h
after birth (n=5, for each phenotype per diet). (g) Histology of back skin of 17d old control and knockout littermates, nursed by mothers fed either control or celecoxib diets. Scale bar: 100 μm. (h) q-PCR analysis from skin biopsies of 15 day old control and knockout mice from litters where nursing mothers were fed control diet or diet containing celecoxib. CT and KO designate control and knockout mice respectively. Bars represent mean values ± S.D. **, p ≤0.01; *, p ≤0.05; NS, not significant.