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Oxidative damage control in a human (mini-) organ: Nrf2 activation protects against oxidative stress-induced hair growth inhibition

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Key Words: Nrf2, Oxidative stress, Lipid peroxidation, Hair growth

Abbreviations: Nrf2 (nuclear factor (erythroid-derived 2)-like 2); SFN (Sulforaphane); ORS (outer root sheath); IRS (inner root sheath); DP (dermal papilla); H_2O_2 (hydrogen peroxide); HO-1 (heme oxygenase-1); NQO1 (NAD(P)H dehydrogenase, quinone 1); ARE (antioxidant response element); GSR (glutathione reductase); GCLC (Glutamate-cysteine ligase, catalytic subunit); GCLM (Glutamate-cysteine ligase, regulatory subunit); GSH (glutathione).

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Abstract

The *in situ* control of redox insult in human organs is of major clinical relevance, yet remains incompletely understood. Activation of Nrf2, the "master regulator" of genes controlling cellular redox homeostasis, is advocated as a therapeutic strategy for diseases with severely impaired redox balance. It remains to be shown whether this strategy is effective in human organs, rather than isolated human cell types. We have therefore explored the role of Nrf2 in a uniquely accessible human (mini-) organ, human scalp hair follicles (HFs). Microarray and qPCR analysis of human HFs following Nrf2 activation using sulforaphane identified the modulation of phase II metabolism, ROS clearance, the pentose phosphate pathway and glutathione homeostasis. Nrf2 knockdown (siRNA) in cultured human HFs confirmed the regulation of key Nrf2 target genes (i.e. HO-1, NQO1, GSR, GCLC, ABCC1, PRDX1). Importantly, Nrf2 activation significantly reduced ROS levels and associated lipid peroxidation. Nrf2 pre-activation reduced oxidative stress-stimulated (H_2O_2 or menadione) premature catagen and hair growth inhibition, significantly ameliorated the H_2O_2-dependent increase in matrix keratinocyte apoptosis and reversed the ROS-induced reduction in proliferation. This study thus provides direct evidence for the crucial role of Nrf2 in protecting human organ function (i.e. scalp HFs) against redox insult.

Introduction

Oxidative stress refers to an imbalance between the production of reactive oxygen species (ROS) and the ability of a cell or tissue to clear this insult. As endogenous ROS production is vital for many cellular signalling processes, including within the HF, a tightly regulated balance between physiological and excess ROS levels needs to be maintained in order to prevent damage to cellular macromolecules (Hamanaka et al. , 2013, Jung and Kwak, 2010, Kloepper et al. , 2015, Schieber and Chandel, 2014). The *in situ* response of human organs to oxidative damage and how they control redox insult is therefore of major physiological and clinical relevance, with oxidative stress associated with a number of human pathologies including chronic kidney disease, neurodegenerative disorders, skin ageing and common dermatoses such as psoriasis (Johnson and Johnson, 2015, Richter et al. , 2015, Wagener et al. , 2013, Zhou et al. , 2009). As such, methods for enhancing antioxidant defences without impeding physiologically important ROS signalling are under investigation (Johnson and Johnson, 2015, Jung and Kwak, 2010, Schäfer and Werner, 2015).

Activation of the transcription factor Nrf2 (nuclear factor (erythroid-derived 2)-like 2), often referred to as the 'master regulator' or redox homeostasis, is a potential therapeutic strategy for diseases with impaired redox balance (Jung and Kwak, 2010); (Clark and Simon, 2009, Clements et al. , 2006). Nrf2 has a recognised role as a multi-organ protector (Moon and Giaccia, 2015, van Poppel et al. , 1999, Wu et al. , 2013), yet the direct study of oxidative damage responses in *human* organs remains challenging.

We have therefore explored the role of Nrf2 in the biology of an accessible human (mini-) organ, namely human scalp hair follicles (HFs), which are frequently exposed to oxidative stress and which represent a prototypic neuroectodermal-mesodermal tissue interaction system (Paus and Cotsarelis, 1999, Schneider et al. , 2009). The current study pursues two distinct goals: a) to exploit human HF organ culture as a uniquely accessible, experimentally tractable and clinically relevant model system (Kloepper et al. , 2010, Philpott et al. , 1990) in which to directly examine the role of Nrf2 in the

responses of a human (mini-)organ to oxidative stress and b) to explore whether Nrf2 up regulation renders human scalp HFs more resistant to oxidative stress. The latter is important, given that a number of human hair growth disorders exists where the enhanced HF resistance to chemical, physical, or pro-inflammatory inducers of oxidative stress would be of major clinical interest (e.g. chemotherapy-induced or radiotherapy-induced alopecia (Paus et al. , 2013, Poeggeler et al. , 2010), alopecia areata (Gilhar et al. , 2012), and UV-induced HF damage (Lu et al. , 2009)). In addition, oxidative stress is likely to play a key role in HF aging (Trüeb, 2015).

We hypothesized that, in the presence of oxidative stress, Nrf2 activation may be an essential factor in protecting the highly proliferative and damage-sensitive matrix keratinocytes of actively growing (anagen) HFs from premature hair growth termination by the induction of apoptosis-driven organ involution (catagen entry) (Bodó et al. , 2007, Paus and Cotsarelis, 1999, Paus et al. , 1994, Schneider, Schmidt-Ullrich, 2009).

Results

Human hair follicles prominently express activated Nrf2

Firstly, the expression and localisation pattern of Nrf2 was determined in human scalp skin and microdissected human HFs. The functional state of intrafollicular Nrf2 was determined using two separate Nrf2-specific antibodies: ab31163 detects both cytosolic (non-activated) and nuclear (activated Nrf2) (Srivastava et al. , 2013), whereas ab76026 detects activated (phosphorylated) Nrf2 (Stachel et al. , 2014).

Nrf2 was prominently expressed throughout the HF epithelium (matrix keratinocytes, inner root sheath (IRS), hair follicle pigmentary unit (HFPU), outer root sheath (ORS, including the companion layer; CL) and bulge region, the dermal papilla (DP) and the mesenchymal connective tissue sheath (CTS) (Figure 1a-c). Ab31163 detected Nrf2 expression in both nuclear and cytosolic compartments (Figure 1a). In contrast, activated Nrf2 (as detected by ab76026) showed high levels of nuclear immunoreactivity (suggesting higher transcriptional activity) in matrix keratinocytes of the anagen VI hair bulb (Figure 1b). Nuclear Nrf2 expression was also found throughout the IRS and ORS (including CL), with lower levels in the CTS and DP. These results show prominent, widespread, but differential intrafollicular expression of Nrf2 and suggest the greatest activity in the anagen hair bulb. This pattern of expression matched that of HFs analysed in human scalp skin biopsies, indicating that the HF dissection and culture process does not alter Nrf2 localisation (Supplementary information, S1, Figure S1).

Nrf2 activation increases the intrafollicular expression of Nrf2 target genes

Intrafollicular Nrf2 transcriptional activity was determined by microarray analysis following treatment with 20 μM sulforaphane (SFN; see Supplementary information S2 for SFN dose response data, Figure S2; GEO accession number: GSE83911).

A large number of genes were significantly up or down regulated (≥2-fold) by SFN. Figure 1D displays some of the most highly regulated genes (n = 3 individual donors). The full list of regulated genes is displayed in Supplementary information Tables S1 and S2. Ingenuity pathways analysis (IPA) indicated that 'NRF2-mediated Oxidative Stress Response' was the top canonical pathway regulated (see supplementary information S3, Figures S3 and S4). A selection of the most highly regulated genes, in addition to known Nrf2 targets (i.e. NAD(P)H quinone oxireductase 1;NQO1, heme oxygenase-1; HO-1, glutathione reductase; GSR) were examined by qPCR most of which showed significant changes (n = 3-6 individual donors; Figure 1e). Nrf2 target genes were also significantly upregulated by a second activator, tert-butylhydroquinone (tBHQ), confirming Nrf2 transcriptional activity (Figure 1f).

A battery of antioxidant defence genes (including those involved in direct ROS clearance, glutathione (GSH) homeostasis and NADPH production) are therefore regulated by Nrf2 activity in human HFs.

Nrf2 activation increases downstream protein expression in human HFs

Confirmation of changes in Nrf2 target protein expression (HO-1, NQO1, peroxiredoxin 1; PRDX1, glutathione reductase; GSR and catalase) as identified by microarray and qPCR analysis, was next performed by immunostaining.

HO-1 immunoreactivity (IR) was extremely low in vehicle treated follicles (Figure 1g) but significantly increased in the CTS following Nrf2 activation by SFN (Figure 1g). Increased HO-1 IR co-localised with the endothelial cell marker CD31 (Supplementary information S4, Figure S5), confirming Nrf2-dependent increases in HO-1 protein in the HF mesenchyme.

NQO1 IR was detected predominantly in the ORS and hair matrix (Figure 1h) and was significantly increased by SFN exposure in this region (Figure 1h). GSR IR was identified throughout the HF epithelium and mesenchyme, and was significantly up regulated in the hair bulb following SFN

activation of Nrf2 (Figure 1j). Both PRDX1 and catalase, showed similar widespread localisation (Figure 1i,k, respectively), yet only PRDX1 IR was increased by Nrf2 activation in the hair bulb (Figure 1i).

The Nrf2-dependence of HO-1 and NQO1 IR was confirmed following tBHQ activation (Supplementary information S5, Figure S6). Due to the high density of cells in the HF matrix, no changes in Nrf2 localisation (nuclear accumulation) following SFN activation were measured (see Supplementary information S6).

Intrafollicular Nrf2 knockdown reduces the ligand-stimulated upregulation of Nrf2 target genes

To further confirm the Nrf2-dependence of antioxidant gene regulation, the impact of Nrf2-silencing on basal and SFN-induced gene expression was investigated in human HFs.

Transfection of organ cultured human HFs with Nrf2 siRNA significantly reduced Nrf2 mRNA levels (approximately 60%) in comparison to HFs transfected with non-targeting scrambled oligonucleotides (SCR) (Figure 2a). Nrf2 siRNA transfection significantly reduced expression of Nrf2 target genes: NQO1, GSR, GCLC, GCLM and PRDX1 (Figure 2b,d,e,f and h, respectively). These genes were significantly up regulated following activation of Nrf2 (SFN), as was ABCC1 (Figure 2b,d,e,f,g and h, respectively). SFN-mediated up regulation of these genes was markedly truncated by Nrf2 siRNA transfection (SFN + siRNA).

Baseline expression of HO-1 was not altered by Nrf2 knockdown, though a small, non-significant increase was stimulated by Nrf2 activation (Figure 2c). HO-1 induction is transiently increased in HFs 6 hours post-SFN stimulation (Supplementary information S2, Figure S2) whereas here, RNA was extracted 24 hours following SFN application, which may have missed expression changes. Neither Nrf2 silencing nor SFN significant altered GPx1 or catalase gene expression (Figures 2i,j), two genes involved in H_2O_2 clearance and previously reported to be Nrf2 targets (Zhu et al. , 2008).

Nrf2 knockdown and activation was similarly analysed in primary cultures of ORS keratinocytes. Modulation of Nrf2 target genes was comparative to intact human HFs (Supplementary information S7, Figure S8). Nuclear translocation of Nrf2 following SFN activation was also confirmed by ICC (Supplementary information S7, Figure S7). Baseline Nrf2 activity (nuclear expression of Nrf2), was lower than is seen in the HF ORS *in situ* (Figure 1a,b), however the regulatory similarities make these cells suitable for determining human HF-related Nrf2 transcriptional responses.

H_2O_2-stimulated ROS production is attenuated by Nrf2 activation

The functional impact of SFN pre-activation of Nrf2 on ROS production following redox stress was determined by cellular DCF fluorescence in ORS keratinocytes. 1 mM H_2O_2, a concentration previously identified to occur within human hair follicles *in vivo* (Wood et al. , 2009), significantly increases ROS production, which was reduced by SFN pre-activation of Nrf2 (Figure 3a). This indicates an increased antioxidant capacity in the ORS keratinocytes following Nrf2 activation**.**

H_2O_2-induced lipid peroxidation is decreased by Nrf2 activation

Lipid peroxidation is a common consequence of excessive ROS levels and can induce apoptosis (Csala et al. , 2015). Changes in lipid peroxidation were therefore determined by 4-hydroxynonenal (4HNE) and acrolein (ACR) IHC in cultured HFs exposed to 1 mM H_2O_2 or blank medium with or without SFN pre-activation of Nrf2 (Figure 3b).

4HNE and ACR IR was substantially increased in the HF bulb by H_2O_2 exposure (Figure 3b), with Nrf2 pre-activation by SFN preventing this increase (Figure 3b). Nrf2 activation therefore reduces oxidative stress and lipid peroxidation in human HFs, which would reduce apoptosis and catagen development (Naito et al. , 2008).

Stress-induced hair growth retardation and catagen induction are attenuated by Nrf2 activation

The functional significance of Nrf2 activity in *ex vivo* human HFs following ROS exposure was determined by measuring hair growth and cycling (see supplementary information S8, for background information). HFs were treated with vehicle (control) or SFN (Nrf2 pre-activation), after which either vehicle or H_2O_2 was added. HFs were maintained in culture for up to 6 days. H_2O_2 exposure significantly reduced hair shaft elongation (Figure 4a). Pre-activation of Nrf2 prior to H_2O_2 restored hair growth rates (Figure 4a). H_2O_2 exposure significantly increased the percentage of regressing (catagen) HFs at day 6 (Figure 4b), which was prevented by SFN pre-activation of Nrf2.

Masson Fontana histochemistry was used to indirectly confirm hair cycle effects (catagen induction results in cessation of intrafollicular melanogenesis (Slominski et al. , 2005)). Melanin distribution was uniform in control HFs, consistent with anagen stage VI. H_2O_2 exposure resulted in a small but significant drop in melanin levels (Figure 4c,d), whereas pre-treatment with SFN maintained a melanin distribution akin to that of untreated control follicles. This independently confirms that SFN prevents H_2O_2-induced catagen induction.

Menadione, which produces superoxide through futile redox cycling, was tested as an additional redox stressor (Braun et al. , 2006). Hair growth was significantly reduced and premature catagen entry increased. This was counteracted by Nrf2 pre-activation, demonstrating protection against excessive superoxide-generation (Supplementary information S9, Figure S9a,b).

Nrf2 activation prevents H_2O_2-induced apoptosis

The impact of redox stress and Nrf2 activation on proliferation and apoptosis in the anagen hair matrix (Schneider et al. 2009), was assessed by quantitative double-immunohistology for Ki67-TUNEL (Hawkshaw et al. , 2015, Kloepper, Sugawara, 2010) (Figure 4e) (additional representative images of Ki67/TUNEL staining can be found in supplementary Figure S10).

H_2O_2 significantly decreased proliferation and increased apoptosis, while SFN pre-activation of Nrf2 prevented this (Figure 4e-g). SFN also prevented a drop in the number of $DAPI^+$ nuclei in the anagen hair matrix following H_2O_2 exposure (Figure 4h), further indication of protection against apoptosis.

The number of $DAPI^+$ cells in the DP stalk provides an instructive indicator of emigrating inductive HF fibroblasts, an indirect read-out parameter for catagen induction (Kloepper, Sugawara, 2010) (see supplementary note S8, for further information). H_2O_2 significantly increased the emigration of DP fibroblasts; whereas SFN significantly reduced this (Figure 4i). H_2O_2 exposure also increased the number of $TUNEL^+$ fibroblasts in the DP and DP stalk, which was again reduced by SFN (Figure 4j,k). As the number of inductive DP fibroblasts are positively correlated with HF size and anagen duration (Hawkshaw, Haslam, 2015, Morgan, 2014, Van Scott and Ekel, 1958), Nrf2 activation may exert long-term effects.

SFN was also found to protect against the inhibition of proliferation following Menadione treatment (Supplementary information S9, Figure S9c-e).

Collectively, these results provide direct evidence that Nrf2 activation prevents reduced hair growth, premature catagen development, and tissue dystrophy caused by oxidative stress in human HFs *ex vivo*, through maintenance of proliferation in the matrix keratinocytes and protection against the loss of inductive fibroblasts from the DP. This demonstrates that Nrf2 activation can exert clinically relevant organ-protective functions in a complex human (mini-) organ.

Discussion

This study provides direct evidence for Nrf2-dependent protection of human HFs against oxidative-stress induced growth retardation and catagen induction (Figure 5). Crucially, these results demonstrate the utility of human HF organ culture as a clinically relevant, accessible and experimentally tractable model system in which to study modulation of redox stress by Nrf2, *ex vivo*.

We have shown that human HFs respond to Nrf2 activation by initiating comparable transcriptional cascades (induction of recognised Nrf2 targets) as reported in other cells and tissues (Bhamre et al. , 2009, Geisel et al. , 2014, Thimmulappa et al. , 2002). In addition, measurement of damage-associated catagen development, a HF regression process driven by apoptosis, provides the most clinically relevant read-out parameter in organ-cultured human HFs, given that premature catagen entry is the chief cause of excessive hair loss (effluvium, alopecia) (Foitzik et al. , 2005, Paus and Cotsarelis, 1999, Paus, Haslam, 2013). Importantly, this provides researchers with a distinctive biological readout with which to monitor the impact of Nrf2 activity in a human *ex vivo* tissue, following exposure to redox-associated stressors.

The protective impact of the Nrf2 transcriptional cascade initiated by SFN exposure is governed by enhanced global antioxidant activity in the HF and the current data set suggests that no single candidate gene is likely to be responsible for catagen-prevention upon ROS exposure. The benefits of Nrf2 activation in the HF and indeed other organs must therefore be considered in holistic terms, considering both up and down regulated genes. Indeed, knockdown of Ly6/PLAUR domain-containing protein 1 (LYPD1), which was down-regulated by SFN in this study, has been reported to increase proliferation (Yu et al. , 2006) whereas metallothionein 1G (MT1G; also down regulated in this study) overexpression can enhance P53 and reduce NFkB signalling (Arriaga et al. , 2014). It is therefore likely that the reduced expression of these genes we identified would be beneficial for anagen maintenance. Likewise, enhanced glutathione recycling and synthesis (GSR, GCLC, GCLM), maintenance of NADPH levels (G6PD, ME1), increased ROS clearance (PRDX1) and cytoprotection

(HO-1) would all serve to shield the HF against the catagen-inducing effects of numerous stressors (Figure 5).

Whereas the HF is commonly believed to be comparatively ROS resistant, continuous exposure to the high levels of ROS produced during intrafollicular melanogenesis could provide one explanation for hair greying, a common sign of HF ageing (Paus, 2011, Paus et al. , 2014, Tobin, 2015). The high proliferative rate of hair matrix keratinocytes and the mitochondrial activity displayed in the human ORS and hair matrix (Vidali et al. , 2014) must also be associated with a continuous exposure to oxidative stress during anagen. Although ROS act as important signaling molecules in the HF, as in other tissues (Hamanaka, Glasauer, 2013, Kloepper, Baris, 2015, Zhao et al. , 2015), this signaling must be finely regulated to prevent damage to cellular macromolecules. Physiologically, the high activity of ROS scavenging enzymes and the local expression of the ROS-protective hormone melatonin, make human HFs generally well equipped to deal with oxidative stress (Kobayashi et al. , 2005, Paus, Langan, 2014).

Yet under pathological conditions, e.g. under chemo- and radiotherapy (Bodó, Tobin, 2007, Poeggeler, Knuever, 2010), stress-related neurogenic HF inflammation (Peters et al. , 2007) and HF overexposure to UVB irradiation (Lu, Fischer, 2009), even the efficient oxidative stress protective system of the HF can become overwhelmed. Excessive ROS accumulation has already been speculated to contribute to HF cell senescence and androgenetic alopecia (Upton et al. , 2015) and the inflammation associated with many of these conditions will also be enhanced by the activity of macrophages/neutrophils, which will release H_2O_2 (Forman and Torres, 2001).

Targeting Nrf2 using an activator such as SFN may therefore represent a promising therapeutic strategy for reducing the damage from excessive accumulation of both endogenous and exogenous ROS. This study demonstrates that utilising *ex vivo* organ cultured human HFs as a model for

studying Nrf2 activity may also provide novel insights into other human diseases associated with oxidative stress.

Materials and Methods

Human hair follicle collection

Occipital scalp hair follicles units were obtained from hair transplant patients. Tissue was obtained following ethical approval (Institute of Inflammation and Repair Ethics Committee, Faculty of Medical and Health Sciences, University of Manchester) and written informed patient consent. Samples were obtained from the Farjo Medical Centre, Manchester, UK.

Human hair follicle organ culture

Human Anagen VI HFs were microdissected and maintained as previously described (Philpott, Green, 1990, Sanders et al. , 1994). For Nrf2 activation, media was prepared with the addition of either vehicle control (DMSO), SFN or tBHQ. Following this treatment, HFs were exposed to redox stressors (1 mM H_2O_2 or 25 µM menadione), at concentrations previously shown to be present in human scalp HFs or utilised to study redox stress *in vitro* (Arck et al. , 2006, Kim et al. , 2014, Sohn et al. , 2007, Wood, Decker, 2009).

ORS keratinocyte isolation and culture and siRNA transfection

ORS keratinocytes (ORSKs) were isolated from male donors (n = 6, median age = 32) as previously described (Borbíró et al. , 2011). At passage 2-3, ORSK cells were plated for siRNA transfection. ORSKs were electroporated by the Neon Transfection System (Life Technologies) using the Neon Kit according to manufacturer's instructions (Borbíró, Lisztes, 2011). Gene expression was assessed using TaqMan probes according to manufacturer's protocol.

Nrf2 knock-down in organ cultured human hair follicles

Dharmacon™ Accell Nrf2 siRNA (SMARTpool: Accell NFE2L2 siRNA ; E-003755-00-0005) or non-targeting controls (Accell Non-targeting siRNA #1; D-001910-01-05) was delivered to isolated human HFs according to the manufacturer's instructions, and not requiring the addition of transfection reagent. HFs were incubated in the presence of Nrf2 or control siRNA for 72 hours. Delivery medium

was removed and HFs were incubated for an additional 24 hours in the presence or absence of SFN. HFs were collected in RNAlater for qPCR analysis. Fold changes in gene expression were compared against the non-targeting controls (SCR).

RNA extraction, microarray analysis and qPCR

Total RNA was extracted from 5 HFs per sample group using the Qiagen RNeasy Microkit (Qiagen) following the manufacturer's instructions. cDNA was synthesised from 250 ng of RNA using the cloned AMV First Strand cDNA Synthesis Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. qPCR was performed using human TaqMan® gene expression assays (Applied Biosystems, Warrington, UK). Reactions were performed and analysed using the StepOne Plus™ Real-Time PCR system and associated software (Applied Biosystems). Relative expression was determined against housekeeping genes (PPIA, GAPDH, ACTB).

Microarray data extracted by the Agilent feature extraction software was analyzed using GeneSpring: data was per sample normalized by percentile shift to the 75th percentile, and baseline transformed to the time-matched vehicle control sample from the same donor. Normalized data was filtered by detection flag and by signal intensity: 20597 gene entities flagged detected and has signal intensity >100 in all replicates of at least 1 experimental condition was used for further analysis by moderated T-test. As few passed Benjamini-Hochberg FDR correction, uncorrected P value cut-off of 0.01 was used: for 24hr treatment with 20uM SFN, 238 genes passed moderated T-test $P<0.01$ >2FC (fold-change) of which 154 were up-regulated and 84 were down-regulated.

Quantitative immunofluorescence

Cryosections (6 µm) were fixed in either 4% PFA (Nrf2, CD31) or acetone (NQO1, HO-1, Catalase, GSR, PRDX1, 4HNE, Acrolein) before permeabilisation with 0.1% triton X (Nrf2, CD31). Washes were performed in phosphate- or tris-buffered saline (PBS, TBS). Primary antibodies were incubated overnight at 4°C before incubation with appropriate alexa fluor secondary antibodies (AF488 or

AF594). Acrolein and 4HNE labelling was performed using the Vector Immpress kits according to manufacturer's instructions (Vector). Staining was visualised using the Vector Immpact Nova Red kit, according to manufacturer's instructions (Vector). Analysis of immunostaining was performed using a Biozero-8000 microscope (Keyence) and staining intensity quantified using ImageJ software (NIH). Masson-Fontana histochemistry and Ki-67/TUNEL double-immunofluorescence microscopy were carried out as described previously (Ito et al. , 2005, van Beek et al. , 2008). Multiple cryosections were analysed from each patient and for each stain in order to account for natural variability.

Materials

Tissue culture reagents were obtained from Gibco (Paisley, UK) or Sigma Aldrich (Gillingham, UK)

Statistical Analysis

Statistical analysis of paired or unpaired Student's t-tests were performed or one-way ANOVA, as appropriate. Bonferroni's post-tests were applied to correct for multiple comparisons.

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Conflicts of Interest

This research was funded by Unilever PLC

Figure Legends

Figure 1. Activation of Nrf2 in human HFs stimulates gene and protein synthesis

(a) Representative images of Nrf2 immunofluorescence in freshly isolated human HFs, detected using the rabbit polyclonal anti-Nrf2 antibody (ab31163). (b) Representative images of active (phosphorylated) Nrf2 immunofluorescence in freshly isolated human HFs, detected using the rabbit monoclonal antibody (ab76026). Scale bar = 50 µm. (c) Cartoon illustrating the salient features of a human HF. (d) Microarray analysis of SFN treated human HFs displaying significant changes in a subset of genes. (e) qRT-PCR analysis (n = 3 separate donors) of genes modulated by SFN in human HFs. (f) qRT-PCR analysis of genes modulated by tBHQ in human HFs. Organ cultured human HFs were treated with vehicle control or SFN for 48 hours. Representative images and quantitative immunofluorescence of the downstream Nrf2 targets (g) HO-1, (h) NQO1, (i) PRDX1, (j) GSR and (k) catalase are shown. Reference areas for quantitation are highlighted in the cartoons to the right of the graphs. Positive control staining in human skin sections is displayed for each target protein. Data are mean +/- SEM of 3-4 separate donors with representative immunofluorescent images shown. Significance relative to untreated controls is indicated by *$p < 0.05$, **$p< 0.01$ and ***$p<0.001$. Scale bar in (a,b,i-k) = 50 µm, scale bar in (g,h) = 100 µm. ORS, outer root sheath; CL, companion layer; CTS, connective tissue sheath, DP; dermal papilla, IRS; inner root sheath, HFPU; hair follicle pigmentary unit, MK; matrix keratinocytes, HS; hair shaft.

Figure 2. Nrf2 siRNA knockdown in human HFs reduces SFN-mediated increases in Nrf2 target gene expression. Organ cultured human HFs were transfected with non-targeting, scrambled oligonucleotides (SCR) or Nrf2 SiRNA (SiRNA) for 72 hours, followed by exposure to vehicle or SFN for a further 24 hours. The mRNA expression of (a) Nrf2 and downstream target genes (b) NQO1, (c) HO-1, (d) GSR, (e) GCLC, (f) GCLM, (g) ABCC1, (h) PRDX1 (i) GPx1 and (j) catalase were assessed by qRT-PCR and reported as fold-changes in normalised expression relative to the

scrambled oligonucleotide controls (SCR). Data are mean +/- SEM of 3-5 individual donors. Significance indicated by *p < 0.05, **p< 0.01 and ***p<0.001.

Figure 3 Nrf2 activation prevents increased lipid peroxidation in HFs by reducing ROS production

(a) ROS production, as measured by increased DCF fluorescence, in human primary ORS keratinocytes following H_2O_2 exposure in the presence and absence of Nrf2 pre-activation by SFN. (b) Representative immunohistochemical staining of lipid peroxidation markers acrolein and 4HNE in human HFs. Data are mean +/- SEM of 3 individual donors. Significance indicated by ***p < 0.001.

Figure 4. SFN prevents H_2O_2 stimulated apoptosis and inhibition of proliferation.

(a) Hair growth (hair shaft elongation) following 6 day culture period in the presence of H_2O_2, SFN or SFN followed by H_2O_2. (b) Hair cycle stage was assessed at the end of the 6 Day culture period, demonstrating a significantly higher proportion catagen follicles in the H_2O_2 treated groups. (c) Representative images of masson fontana histochemistry. (d) Analysis of masson fontana histochemistry. (e) Representative images of Ki67/TUNEL double staining in the bulb region of isolated hair follicles treated with vehicle control, H_2O_2, SFN and SFN + H_2O_2. (f) Percentage of $Ki67^+$ and (g) $TUNEL^+$ cells in the proliferative matrix keratinocytes. (h) Number of DAPI positive cells below Auber's line (white dashed line), representing an indirect marker of apoptosis. (i) Number of DAPI positive cells in the DP stalk, representative of emigrating fibroblasts. (j) Number of $TUNEL^+$ cells in the DP stalk and (k) in the DP. Data are mean +/- SEM of 4-6 individual donors. Significant differences between the H_2O_2 and SFN + H_2O_2 groups in (a) are represented by *p < 0.05 and **p < 0.01. Significant differences between the Control and H_2O_2 groups in (a) are represented by ##p < 0.01 and ###p < 0.001. Significance is represented in all other panels by *p < 0.05, **p < 0.01 and ***p<0.001. Scale bars = 50 μm. HFPU, hair follicle pigmentary unit.

Figure 5. Schematic representation of Nrf2-mediated protection in human HFs

Activation of Nrf2 (i.e. by SFN) will induce the expression of a host of downstream targets genes encompassing pathways responsible for the reduction of reactive quinones, direct ROS clearance, GSH homeostasis, cytoprotection and NADPH production. The net effect is to reduce or limit ROS production and associated damaging effects, effectively reducing apoptosis and catagen induction.

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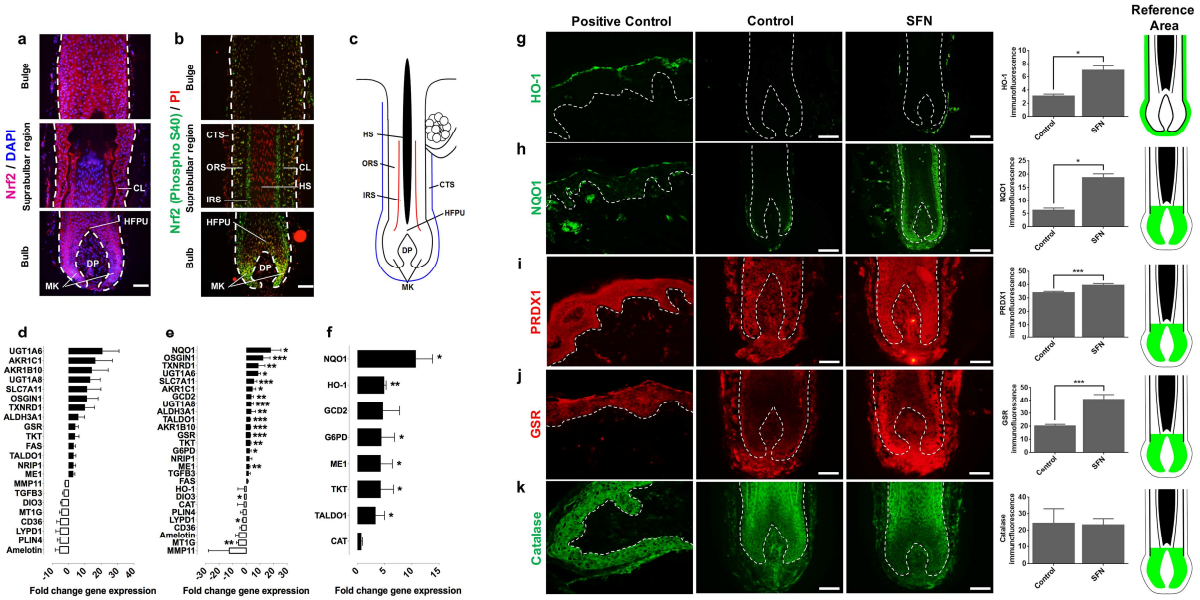

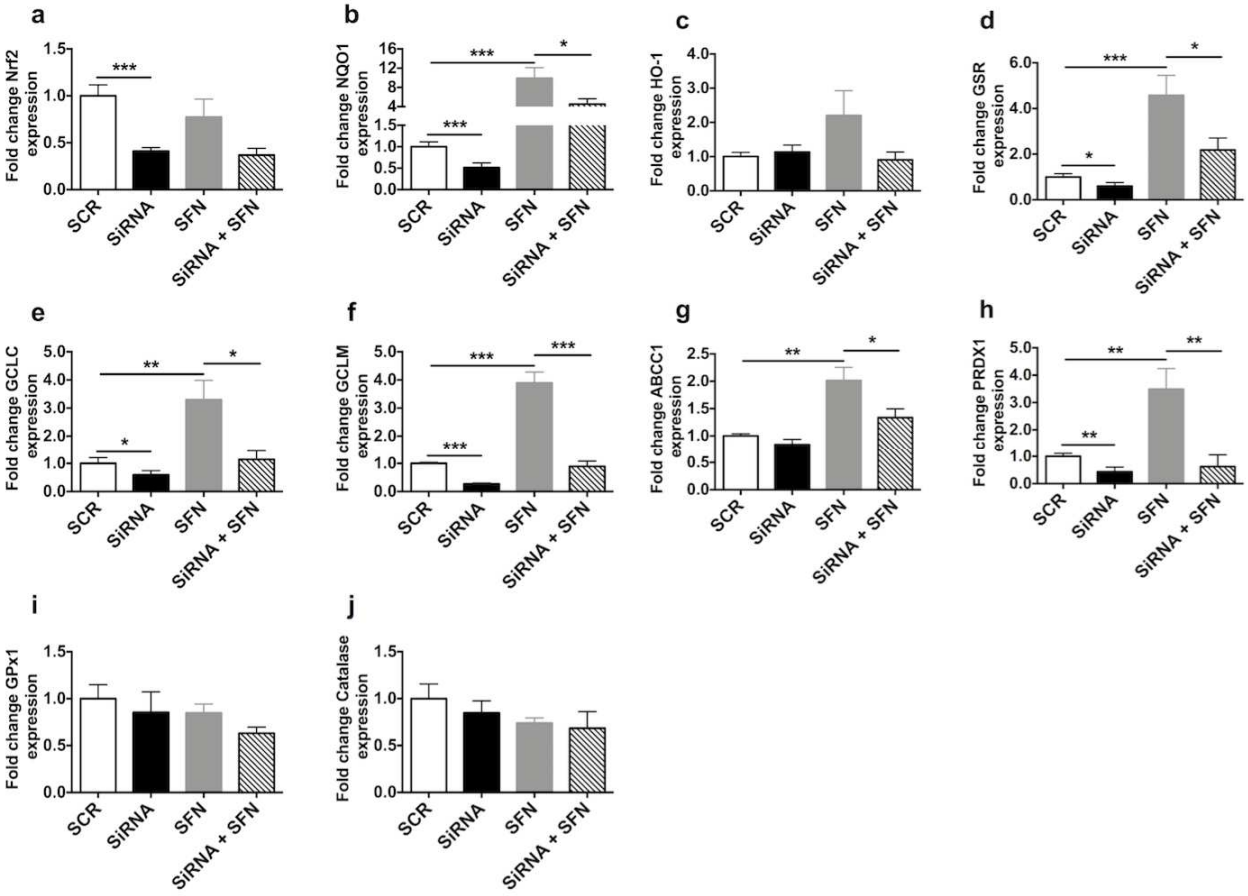
a
Fold change Nrf2 expression
b
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c
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d
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e
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SCR
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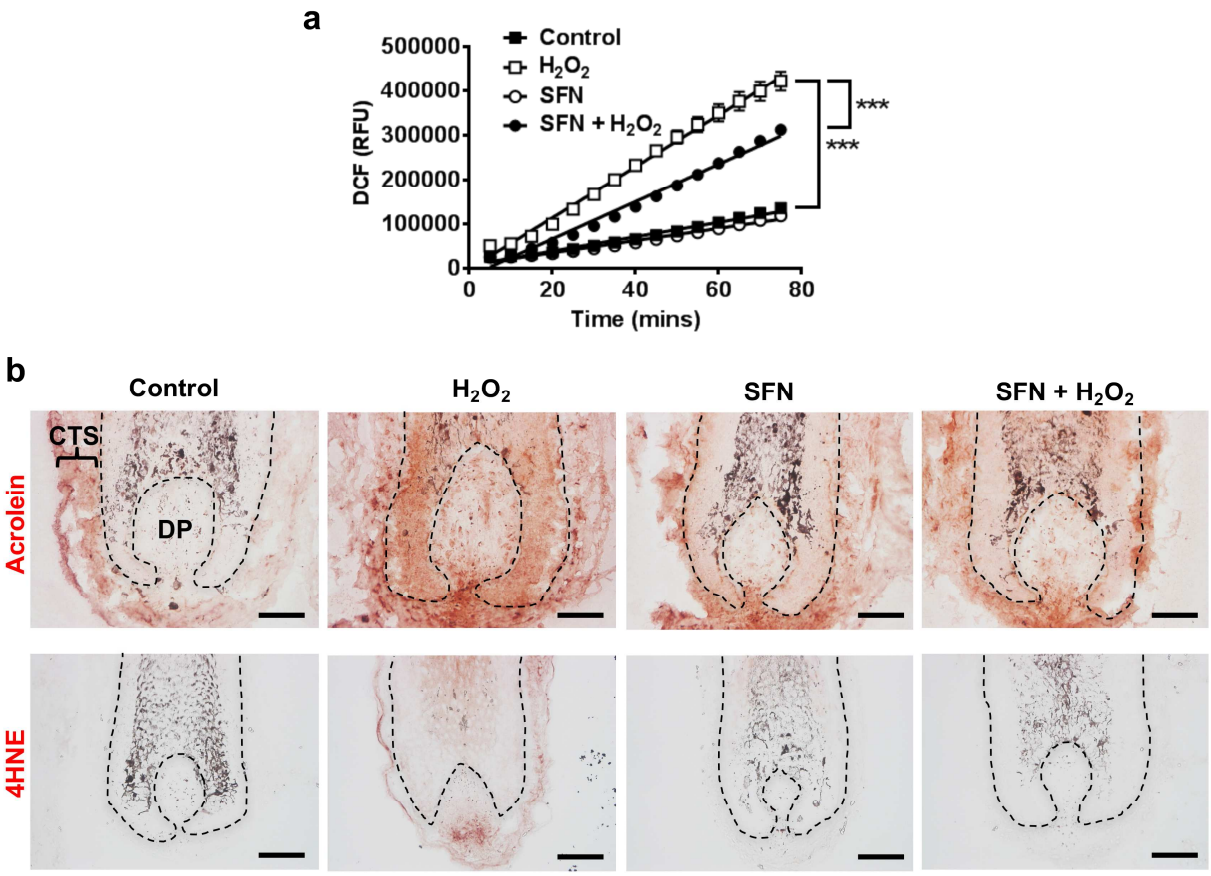
a
Control
H2O2
SFN
SFN + H2O2
DCF (RFU)
500000
400000
300000
200000
100000
0
0
20
40
60
80
Time (mins)
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b
Control
H2O2
SFN
SFN + H2O2
CTS
DP
Acrolein
4HNE