Abstract

In order to model squamous cell carcinoma development in vivo, researchers have long preferred hairless mouse models such as SKH-1 mice that have traditionally been classified as ‘wild-type’ mice irrespective of the genetic factors underlying their hairless phenotype. The work presented here shows that mutations in the Hairless (Hr) gene not only result in the hairless phenotype of the SKH-1 and Hr-/- mouse lines but also cause aberrant activation of NFκB and its downstream effectors. We show that in the epidermis, Hr is an early UVB response gene that regulates NFκB activation and thereby controls cellular responses to irradiation. Therefore, when Hr expression is decreased in Hr mutant animals there is a corresponding increase in NFκB activity that is augmented by UVB irradiation. This constitutive activation of NFκB in the Hr mutant epidermis leads to the stimulation a large variety of downstream effectors including the cell cycle regulators cyclin D1 and cyclin E, the anti-apoptosis protein Bcl-2, and the pro-inflammatory protein Cox-2. Therefore, Hr loss results in a state of uncontrolled epidermal proliferation that promotes tumor development, and Hr mutant mice should no longer be considered merely hairless ‘wild-type’ mice. Instead, Hr is a crucial UVB response gene and its loss creates a permissive environment that potentiates increased tumorigenesis.

Introduction

As a group, non-melanoma skin cancers (NMSC) are the most common type of cancer in the US with a combined incidence of over 2 million new cases annually [1]. Squamous cell carcinoma (SCC) is the second most common form of NMSC and is the most likely type to lethally metastasize, which makes understanding SCC development crucially important to human health [2]. In recent years, several genetic pathways have been implicated in the events leading to SCC development and progression remain poorly understood. Current research strategies utilize mice strains that lack hair to model carcinogenesis using either UV irradiation or chemical carcinogenesis since researchers prefer not to perform repeated shaving or depilation to visualize tumor development. Particularly favored strains feature mutations in the Hairless (Hr) gene (Entrez Gene ID 15460) that result in almost complete hair loss early in life [6].

Hairless mutant mice first appeared in the literature in the 1930’s when they were described as having a striking wave of hair loss that occurs in a rostral to caudal manner starting at 3 weeks of age [6,7,8,9]. Some fifty years later, Hr mutant mice were widely adopted as a convenient model for skin carcinogenesis studies and were discovered to be uniquely susceptible to both UV induced and chemical carcinogenesis [10,11,12]. Since then, the molecular basis of the Hairless phenotype has been established as either a proviral insertion into exon 6, as in the case of Hr<sup>os</sup> and SKH-1 animals [13,14], or point mutations, as is the case of the Hr<sup>Os</sup> animals [15,16,17]. Despite the knowledge that these strains have a mutated Hr gene, many carcinogenesis studies continue to use them and refer to unirradiated/untreated animals as ‘wild-type hairless’ mice.

Two early experiments using different Hairless mutant animals suggested that the increased susceptibility to carcinogenesis was, in fact, due to disruption of Hr. First, Iversen and Iversen (1976) showed increased tumorigenesis in Hairless mice bearing the Hr/Hr Oslo mutation as compared to wild-type, hairy littermates when both cohorts were exposed to a chemical carcinogenesis protocol. Secondly, Stoye et al. (1988) described the appearance and propagation of a revertant, hairy mouse (Hr<sup>Rev</sup>) that spontaneously lost the proviral insertion in the Hr allele. When used in both chemical and UV induced carcinogenesis protocols, the Hr<sup>Rev</sup> mice were found to develop less tumors as compared to their Hr<sup>os</sup> counterparts [11,18]. Taken together, these genetic data suggested that Hairless is a key determinant of skin tumorigenesis.
Due to the overall phenotype of Hairless mutant mice, it has long been hypothesized that Hairless plays crucial roles in both the interfollicular epidermis and hair follicle. Supporting this hypothesis is the fact that Hr has been shown to be abundantly expressed in the suprabasal layers of the interfollicular epidermis, the matrix region of the hair follicle in catagen, and other follicular compartments such as the dermal papillae [19]. We (and others) have established a role for Hairless in the regressing catagen hair follicle [20,21,22], yet its function in the interfollicular epidermis has remained elusive. What has been deduced so far is that Hairless functions as a transcriptional co-factor that interacts with several nuclear hormone receptors such as the Vitamin D receptor (VDR) and thyroid hormone receptor (TR) (reviewed in [23]). In several nuclear hormone receptors such as the Vitamin D receptor (VDR) and thyroid hormone receptor (TR) (reviewed in [23]), Hairless functions as a transcriptional co-factor that interacts with multiple domains raises the possibility that Hr regulates multiple pathways thereby having pleotropic effects in the epidermis and influencing tumor susceptibility.

UV induced tumorigenesis occurs, in part, through the activation of the NFκB pathway and subsequent cellular stress and inflammation, therefore, one of the key UV response mechanisms is regulation of the NFκB pathway (reviewed in [24]). Furthermore, constitutive activation of the NFκB pathway is a hallmark of several types of tumors including SCCs [25,26,27], while its inhibition is a known chemoprevention for several tumor types (reviewed in [28]). Recent work from our lab has shown that in primary human keratinocytes HR is an early UVB response gene that regulates UVB induced NFκB activation thereby modulating cell cycling [29]. Due to these in vitro results and the clear role of the NFκB pathway in UV induced carcinogenesis, we postulated a connection between Hr and the NFκB pathway in vivo. Here, we present multiple lines of evidence demonstrating that disruption of the Hr gene leads to constitutive activation of the NFκB pathway, uncovering the molecular mechanism of the skin tumorigenesis susceptibility of Hairless mutant mice for the first time.

Results

Hr is rapidly upregulated in response to UVB and loss of Hr increases susceptibility to UVB induced tumorigenesis

In order to place our studies in the context of earlier work, we first confirmed that the mouse lines SKH-1 and Hr+/ (hereafter referred to as Hr+/−) both contained the mutation, a proviral insertion disrupting the open reading frame of Hr (Figure S1). We then quantified and compared the level of Hr mRNA from whole skin extracts of Hr+/−, SKH-1, and WT littermates. Both Hr+/− and SKH-1 animals exhibited significantly lower levels of Hr mRNA that equaled an ~70% decrease in expression (Figure 1). Our previous work showed that Hr is expressed in suprabasal keratinocytes [19,20] and is involved in the early UV response mechanism in primary human keratinocytes [29]. Therefore, we decided to focus our future experiments on the epidermis. In order to implicate Hr in the UV response pathway in vivo, we used real time PCR to evaluate Hr levels in the WT (Hr+/+) epidermis at baseline as compared to 1, 8, and 24 hrs after a single, acute UVB dose. We observed a three-fold increase in epidermal Hr mRNA transcript levels from baseline (3.028±0.6147) as early as 8 hrs post UVB exposure (Figure 1B). Thus, Hr is rapidly transcriptionally activated in response to acute UVB suggesting that Hr plays a role in the immediate UVB response.

To assess the impact of Hr mutations to UVB response, we performed a long-term UVB tumorigenesis protocol [180 mJ/cm², the equivalent of 2 MEDS [30,31], three times a week for a total of 50 weeks] using Hr+/− and strain-matched WT (Hr+/+) animals. Neither WT (Hr+/+) nor Hr+/− animals showed any signs of papilloma formation at 22 weeks, however by week 30, Hr+/− animals began to develop small papillomas (Figure S2). 50 weeks into the protocol, the Hr+/− animals had an average of 22 tumors that necessitated the termination of the study (Figure 2). In contrast, WT (Hr+/+) animals showed no sign of tumor or papilloma formation at any time during the study.

Histological analysis of tissues obtained from the tumors in Hr+/− animals at 50 weeks indicated that they were highly aggressive SCCs, while there was no evidence of even microscopic tumor formation in WT (Hr+/+) skin (Figure 2B). Since the only known genetic difference between Hr+/− animals and WT (Hr+/+) littermates was the Hairless mutation (Figure 1), these results demonstrate that the disruption of Hairless was directly responsible for conferring tumor susceptibility.

The Hairless mutant epidermis is hyperproliferative both basally and in response to UVB

Given the evidence in the literature and the results of our long-term UVB study, we next investigated the molecular role of Hr in the UVB response pathways. For these studies we used a single, acute UVB dose (180 mJ/cm²) and assessed early time points (1, 8,
and 24 hrs post-irradiation) without the variable of secondary effects of a long term UVB protocol.

Using epidermal morphometry, we found a significant increase in epidermal thickness both before and after UVB in \( Hr^{-/-} \) mice as compared to wildtype counterparts (Figure 3A). At baseline, \( Hr^{-/-} \) mice have a significantly thicker epidermis indicating the possibility of a hyperproliferative phenotype. Increased epidermal thickness in \( Hr^{-/-} \) skin is maintained after UVB exposure with gradual increases at the indicated timepoints. We hypothesized that such an increase in epidermal thickness was due to either increased numbers of proliferating keratinocytes or a defect in apoptosis.

Therefore, we investigated the amount of proliferation found in the Hairless mutant epidermis by staining skin sections from \( Hr^{-/-} \) and their respective WT littermates with the proliferation marker Ki67. As seen in Figure 3B, the percent of Ki67 positive cells found in the non-irradiated \( Hr^{-/-} \) epidermis was significantly higher than in the WT epidermis [0.1969 ± 0.0246 (WT) vs 0.4649 ± 0.0772 (\( Hr^{-/-} \)) as measured by the amount of Ki67 positive basal cells as a percentage of total basal cells]. Throughout
the time points sampled, the \( Hr^- \) epidermis continued to exhibit a higher amount of proliferation than WT epidermis. Altogether this data indicated that the epidermal thickening seen in Figure 3A may, in part, be due to increased proliferation in the Hairless mutant epidermis.

In order to determine if the epidermal thickening could be partially attributed to defects in apoptosis, TUNEL staining was also performed (Figure 3C). We found that at homeostasis both WT and \( Hr^- \) epidermis contained a low level of apoptosis that increased 1 hr after UVB exposure. However, the amount of apoptosis found in the \( Hr^- \) epidermis was approximately 5 times less than in WT controls. This trend continued at 8 and 24 hrs with the level of apoptosis in the \( Hr^- \) epidermis always being significantly less than that found in WT epidermis. When taken together with the proliferation data, this suggests that apoptosis may be impaired by loss of \( Hr \) perhaps due to signals favoring proliferation or possibly because of the increased epidermal thickness partially shielding the keratinocytes from UVB irradiation.

**Loss of \( Hr \) leads to constitutively activated NF\( k \)B**

NF\( k \)B has long been known to play a role in UVB induced tumorigenesis and forms a nexus of proliferative and apoptotic pathways; additionally, our previous work shows that HR regulates NF\( k \)B in vitro [29]. We therefore theorized that \( Hr \) and NF\( k \)B may be mechanistically related in a similar fashion in vivo. To investigate a possible connection between the two proteins, we examined p65 protein levels in \( Hr^- \) and matching WT (\( Hr^+/+ \)) littermate epidermis with and without acute UVB irradiation using Western blotting (Figure 4A). Prior to irradiation, \( Hr^- \) epidermis had a significantly higher amount of the p65 subunit of NF\( k \)B. \( Hr^- \) epidermis continued to express higher levels of p65 at 1 hr, 8 hrs, and 24 hrs post-UVB irradiation. Altogether this indicates that the lack of \( Hr \) in the \( Hr^- \) epidermis results in a constitutively upregulated NF\( k \)B pathway.

In order to determine if the increase in p65 protein levels correlated with activation of the NF\( k \)B signaling pathway following acute UVB exposure, we examined \( Hr^- \) and WT (\( Hr^+/+ \)) skin sections using an antibody against the phosphorylated inhibitor of NF\( k \)B signaling, I\( k \)B\( \alpha \). Activation of the NF\( k \)B complex is dependent on I\( k \)B\( \alpha \) phosphorylation (p-I\( k \)B\( \alpha \)) and subsequent degradation that allows nuclear translocation of the NF\( k \)B complex [32]. In addition to being upstream of NF\( k \)B, I\( k \)B\( \alpha \) is also a known target gene that is transcriptionally regulated by NF\( k \)B [32]. Therefore, NF\( k \)B and I\( k \)B\( \alpha \) form a positive feedback loop and p-I\( k \)B\( \alpha \), when assessed in conjunction with the overall increased levels of p65, can be used as an indicator to specifically localize the activated NF\( k \)B pathway [33].

Staining of non-irradiated \( Hr^- \) skin showed the presence of p-I\( k \)B\( \alpha \) throughout all the epidermal layers, including the basal layer, while in WT (\( Hr^+/+ \)) skin I\( k \)B\( \alpha \) expression was restricted to the differentiated, suprabasal layers (Figure 4B). This clear demarcation of p-I\( k \)B\( \alpha \) expression suggests that perturbation of NF\( k \)B signaling in basal keratinocytes may be responsible for the dysregulated proliferation and apoptosis observed in Hairless mutants. In response to acute UVB, we observed a gradual increase in the levels of p-I\( k \)B\( \alpha \) in WT (\( Hr^+/+ \)) skin, with signaling in the basal keratinocytes beginning at 8 hrs post-UVB irradiation (Figure 4B). This expression pattern coincides with the time point previously observed to have a significantly higher amount of \( Hr \) mRNA in the WT epidermis after acute irradiation (Figure 1B). As expected, the intensity of p-I\( k \)B\( \alpha \) staining increased in response to UVB exposure in both the WT (\( Hr^+/+ \)) and \( Hr^- \) mutant skin and by 24 hrs post UVB irradiation, both genotypes showed uniform p-IkB\( \alpha \) staining in all layers of the epidermis with comparable intensity.

To study the role of NF\( k \)B activation in the increased susceptibility of Hairless mutant mice to tumorigenesis, we returned to the non-tumor bearing skin samples generated in the long-term irradiation study described in Figure 2. Interestingly, p-IkB\( \alpha \) staining appeared in the basal and suprabasal layer of the mutant epidermis by week 25, a time point prior to tumor development (Figure 4C). In contrast, at the same timepoint, the WT (\( Hr^+/+ \)) epidermis continued to show only suprabasal localization of p-IkB\( \alpha \). By week 30 (when tumors were first observed on \( Hr^- \) mice) the staining pattern remained the same in both sets of animals, though the \( Hr^- \) epidermis had a higher intensity of staining. The intensity of the p-IkB\( \alpha \) staining in the \( Hr^- \) epidermis continued to increase at week 42 and plateaued at week 50, while the WT (\( Hr^+/+ \)) epidermis only began to show staining in the basal layer of the epidermis at week 42 which increased at week 50.

**Pharmacological inhibition of NF\( k \)B signaling reduces UVB induced tumorigenesis in Hairless mutant mice**

We next reasoned that blockade of NF\( k \)B signaling in vitro should inhibit tumor formation in Hairless mutant mice. Therefore, we performed chronic UVB tumor induction studies while simultaneously treating the mice with a pharmacological inhibitor of NF\( k \)B, pyrrolidinedithiocarbamate (PDTC), which was given ad libitum the mice’s drinking water. PDTC suppresses NF\( k \)B DNA-binding activity and I\( k \)B\( \alpha \) degradation thereby inhibiting NF\( k \)B activation [34]. 60 SKH-1 mice were divided into two groups: group-I mice received tap water and group-II mice received PDTC (0.5%) supplemented drinking water. All mice were irradiated with UVB, 180 mJ/cm\(^2\) twice a week for 35 weeks, which was sufficient to induce tumorigenesis both with and without PDTC treatment (Figure 5A and B).

PDTC and its concomitant reduction in NF\( k \)B activity significantly reduced tumorigenesis by approximately 60%, as measured in average tumor number, throughout the duration of the irradiation protocol (Figure 5A). Average tumor volume was also significantly decreased in the animals treated with PDTC (Figure 5B). Inhibition of the NF\( k \)B pathway altered gross tumor morphology suggesting that the tumors that formed on the PDTC treated animals were more likely to be papillomas than SCCs. In contrast, the animals given only tap water developed signs of aggressive SCCs (Figure S3A). Taken together, these results demonstrate that the tumor susceptibility of the SKH-1 animals is dependent on the overactivation of the NF\( k \)B pathway that is a downstream result of Hairless mutation.

We next performed a detailed molecular analysis of the NF\( k \)B pathway in the PDTC treated SKH-1 animals (Figure 5C-F). For this analysis we used the non-tumor bearing skin and the tumors from the PDTC treated and control SKH-1 animals that had been irradiated for 35 weeks. In addition to analyzing whole tumors in this study, the epidermis was isolated from non-tumor bearing skin in order to localize the biochemical effects of the PDTC treatment. Our initial hypothesis was that non-tumor bearing skin of the PDTC treated animals would exhibit reduced NF\( k \)B activation in the increased \( Hr \) levels of the epidermis after acute irradiation study described in Figure 2. Interestingly, p-IkB\( \alpha \) staining appeared in the basal and suprabasal layer of the mutant epidermis by week 25, a time point prior to tumor development (Figure 4C). In contrast, at the same timepoint, the WT (\( Hr^+/+ \)) epidermis continued to show only suprabasal localization of p-IkB\( \alpha \). By week 30 (when tumors were first observed on \( Hr^- \) mice) the staining pattern remained the same in both sets of animals, though the \( Hr^- \) epidermis had a higher intensity of staining. The intensity of the p-IkB\( \alpha \) staining in the \( Hr^- \) epidermis continued to increase at week 42 and plateaued at week 50, while the WT (\( Hr^+/+ \)) epidermis only began to show staining in the basal layer of the epidermis at week 42 which increased at week 50.
irradiated SKH-1 animals to be more prominent in the non-tumor bearing skin compared to non-treated SKH-1 animals.

Figure 4. *Hairless* mutant epidermis shows constitutive activation of the NFκB signaling pathway. A) Western Blot and ImageJ densitometry analysis using anti-p65 antibody on isolated *Hr*−/− epidermal extracts after 1, 8 and 24 hrs post UVB (180 mJ/cm²) irradiation. B) Immunohistochemistry using p-IκBα antibody to determine the extent of NFκB signaling activity in the epidermis of WT (*Hr*+/+) and *Hr*−/− animals before and after a single, acute UVB (180 mJ/cm²) irradiation. IκBα is increased constitutively and after irradiation throughout the epidermal layer. C) Immunohistochemistry using p-IκBα antibody to determine the extent of NFκB signaling activity in the epidermis of WT (*Hr*+/+) and *Hr*−/− animals at the indicated time points during chronic UVB irradiation (180 mJ/cm² UVB three times a week for a total of 50 weeks). IκBα is increased constitutively and after irradiation throughout the epidermal layer.

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*PDTC inhibition of NFκB blocks downstream target genes in chronically UVB exposed Hairless mutant skin*

We began by assessing NFκB activation by Western blot analysis of nuclear extracts; we found decreased nuclear localiz-
tion of the NFκB subunits p65 and p50 in non-tumor bearing epidermis and tumors but no change in p52 (Figure 5C). This indicated that as expected, PDTC specifically blocked nuclear translocation of the p65/p50 heterodimer and the canonical NFκB pathway. We also found that PDTC treated epidermis exhibited decreased NFκB activation as measured by direct binding of NFκB to consensus binding sites, electrophoretic mobility shift assay (EMSA) (Figure S3B). Total and phosphory-
lated IkBα protein levels were measured by Western blot in non-tumor bearing epidermis and tumors; it was found that PDTC treatment specifically abrogated increases in phosphorylated IkBα in non-tumor bearing skin suggesting a functional blockade of the NFκB signaling pathway (Figure S3C). Taken together, the data indicates an overall reduction in NFκB signaling activity in the non-tumor bearing skin of SKH-1 animals subjected to chronic UVB exposure and treated with PDTC.

In order to assess the downstream consequences of NFκB regulation by PDTC in SKH-1 animals, we analyzed the mRNA expression of various NFκB target genes in epidermal extracts and tumors using semi-quantitative PCR. As shown in Figure 5D, we found that PDTC treatment significantly reduced the expression of TRAIL [35], IL-1β [36], TNFα [37], and MnSOD [38] in the non-tumor bearing epidermis with a less appreciable decrease in tumors. This data confirms that NFκB activation is crucial for tumor development in chronically UVB irradiated SKH-1 skin, and that PDTC efficiently blocked NFκB activation in non-tumor bearing skin, thus attenuating the susceptibility of SKH-1 animals to tumor development.

To further investigate the PDTC-dependent repression of tumor susceptibility in chronically irradiated Hairless mutant mice, we studied its effects on the expression of genes that regulate cell cycle progression, proliferation, and apoptosis. As shown in Figure 5E, PDTC treatment inhibited the expression of the key cell cycle regulators cyclin D1 [39], cyclin E1 [40], and PCNA [41] in both tumors and in non-tumor bearing epidermis. When combined with the increased Ki67 staining seen in Figure 3B, increased expression of these proteins in irradiated SKH-1 skin and tumors is yet another indication of a hyperproliferative state that can be abrogated by blockade of the NFκB pathway.

We then examined the expression of two proteins involved in apoptotic response pathways, the pro-apoptotic caspase 3 and the anti-apoptotic Bcl-2. Caspase 3 has not been implicated in the NFκB pathway so as expected, we saw no significant change in expression with PDTC treatment (Figure 5F). Bcl-2, however, is a known NFκB target gene and its expression is decreased with PDTC treatment in both non-tumor bearing epidermis and tumors (Figure 5F) [42]. This is consistent with the TUNEL and Ki67 results in Figures 3B and 3C that show increased proliferation and decreased apoptosis, which together with the data presented here, illustrates that repression of NFκB restores the normal apoptotic response in the irradiated Hr mutant epidermis.

To further understand the mechanism behind increased tumor promotion in the context of NFκB activation, we assessed the expression of the pro-inflammatory protein COX-2. COX-2 is known to be downstream of NFκB and is strongly implicated in squamous cell carcinoma progression [43,44,45]. In agreement with the expression pattern of the other pro-inflammatory NFκB target genes TNFα and IL-1β, we found that COX-2 levels were dramatically decreased with PDTC treatment in non-tumor bearing skin but not in tumors. Therefore, we theorize that in the Hr mutant epidermis, increased COX-2 levels in conjunction with TNFα and IL-1β create an pro-inflammatory environment that promotes tumor growth [46].

**Discussion**

Hairless mice, particularly the SKH-1 line, have long been a favorite animal model used for skin carcinogenesis studies because of their convenience, however, researchers have not generally taken into consideration the underlying mechanism for their lack of hair and whether this may be linked to their tumor susceptibility. To address this issue, we show for the first time that Hr−/− and SKH-1 mouse lines are allelic mutants of one another that have significantly lower amounts of Hr, a crucial early UVB response gene, and are thus predisposed to UVB induced carcinogenesis. The results detailed above indicate that Hr plays a crucial role in cell cycle kinetics by functioning as an inhibitor of the NFκB pathway in the epidermis; therefore, loss of Hr results in altered cell cycling, increased proliferation and subsequent tumorigenesis.

We found that both Hr−/− and SKH-1 animals exhibit similar decreases of Hr expression throughout the skin that amounts to approximately 30% of that found in WT animals; this allowed them to be used interchangeably in our experiments. We further determined that Hr is significantly upregulated in the WT epidermis 8 hours after a single, acute dose of UVB. Furthermore, when Hr−/− mice were used in chronic UVB carcinogenesis studies they developed papillomas by week 30, and these papillomas progressed into aggressive SCCs by the end of the protocol 20 weeks later. In contrast, WT mice with normal Hr expression failed to show even microscopic signs of tumorigenesis by week 30.

Together, these findings led us to postulate that Hr is an essential component of the epidermal response to UVB insult and perturbation of Hr levels causes a genetic predisposition to tumorigenesis due to a dysregulated UVB response in the Hr mutant epidermis. Under normal conditions, a tightly regulated signaling cascade is triggered by UVB irradiation leading to the apoptosis of severely damaged cells and proliferation of undamaged and/or repaired cells (reviewed in [47]). Therefore, we examined the epidermis of Hr−/− animals and found that at homeostasis Hr loss results in a significantly thicker epidermis that is sustained after UVB exposure. We then investigated the contribution of both proliferation and apoptosis to this thicker epidermis and found both increased proliferation at all time points and decreased apoptosis in response to UVB irradiation. This indicated that the thicker epidermis found in Hr mutant mice was the result of uncontrolled proliferation and decreased apoptosis, factors which when combined together contribute to the increased tumorigenesis seen during the chronic UVB exposure protocol. It should be noted that the thicker epidermis in and of itself might contribute to the decreased apoptosis due to possible UVB shielding.

One of the key mechanistic links between proliferation and apoptosis is the NFκB signaling pathway and we have previously established a regulatory loop between Hr and NFκB in vitro [29,46]. Thus we investigated a possible mechanistic connection between NFκB and Hr in vivo. While there are many NFκB hetero- and homo-dimers that can activate NFκB-mediated transcription and regulate cell proliferation, we began by focusing on the canonical p65/p50 hetero-dimer complex since it is known to be an important UVB response factor in both mouse and human skin [24]. We found that at homeostasis, Hairless mutant epidermis exhibits constitutive activation of the NFκB pathway as indicated by increased expression of both the p65 subunit and p-IκBα. Unlike in the WT epidermis, p-IκBα activation in the Hr−/− skin is not confined to the superficial layer; instead p-IκBα is expressed throughout the epidermis both basally and in response to UVB thereby indicating aberrant upregulation of NFκB in Hr−/− skin.

We postulate that Hr regulates tumor promotion via regulation of NFκB activation and thereby modulates proliferation and apoptosis following UVB irradiation. Therefore, when Hr expression is decreased, there is an increased likelihood of the survival and proliferation of UVB damaged basal keratinocytes and subsequent tumorigenesis. As we also show here, blockade of
the NFκB pathway by the pharmacological inhibitor PDTC decreases both tumor burden and tumor volume in the Hairless mutant mouse line SKH-1.

In the non-tumor bearing skin irradiated SKH-1 mice, PDTC treatment specifically decreased NFκB activity in the Hairless mutant epidermis as reflected by attenuated expression of the NFκB subunits p50 and p65, decreased NFκB binding to target sequences, and decreased expression of p-IκBα. Additionally, the non-tumor bearing skin of PDTC treated mice also exhibited decreases in the expression of downstream NFκB targets such as TRAIL, IL-1β, TNF-α, and MnSOD as well as the inhibition of cell cycling proteins such as cyclin D1, cyclin E, PCNA, and BCL-2 thereby indicating a return to normal proliferation. We also show that in non-tumor bearing skin, Hr influences inflammation via COX-2 expression which decreases with PDTC treatment. The combination of decreased TNF-α, IL-1β, and COX-2 found in the PDTC treated epidermis provides compelling evidence that Hr is capable of modulating epithelial inflammation via regulation of the NFκB pathway and thereby further contributes to tumor promotion. Therefore, the data provides evidence that the decreases in Hr expression result in gross epithelial dysregulation that can be ameliorated by inhibition of the NFκB pathway.

We hypothesize that since NFκB is the nexus of proliferative and inflammatory signals [46], the lack of Hr allows the proliferation of UVB damaged cells and eventual tumor development. Previous work in our laboratory has shown that after UVB insult, cells lacking Hr have decreased amounts of cells in the G2/M phase [29] which is a key cell cycle checkpoint for DNA damage [48,49]. Attenuation of the G2/M checkpoint after UVB insult increases genomic instability which when combined with increased proliferation and inflammation found in the Hairless mutant epidermis creates an ideal cellular environment for tumor development. Thus, loss of Hr and subsequent overactivation of NFκB provide all the components necessary for increased tumorigenesis.

While it is possible that Hr-mediated repression of the NFκB pathway could occur at multiple junctures due to the inherent complexities of the NFκB pathway, the evidence presented here suggests the involvement of Hr in two possible regulatory mechanisms. One is through the control of the kinase cascade leading to IκBα phosphorylation and subsequent degradation. This then allows the canonical NFκB heterodimer to translocate to the nucleus, bind to target DNA sequences, and modulate downstream gene transcription. This possibility is seen in the increased IκBα staining seen in Hr−/− epidermis both prior to and after irradiation. The other possible regulatory mechanism is through direct transcriptional regulation of the p65 subunit. Evidence for this regulation is seen in the increased expression of p65 in epidermal extracts from unirradiated and irradiated Hr−/− mice. Since IκBα is also downstream of NFκB activation, direct transcriptional regulation of the NFκB subunits would also result in the increased IκBα phosphorylation seen in the Hr mutant epidermis. Therefore, either hypothesis fits the data presented here and further work will be necessary to clarify the method of Hr regulation of NFκB.

While non-tumor bearing skin showed significant changes with PDTC, there were less obvious effects in the tumor samples. We postulate that specific keratinocyte populations escaped PDTC-mediated inhibition of NFκB and developed into tumors despite PDTC treatment. We further postulate that this escape may be due to other molecular events such as DNA damage in other genes downstream of PDTC inhibition of the NFκB pathway. Therefore, the effects of PDTC are more prominent in the non-tumor bearing skin and a small number of tumors develop despite PDTC treatment.

Altogether, we provide here multiple lines of evidence that Hr plays an essential role in the regulation of NFκB signaling in the epidermis and thereby effects keratinocyte apoptosis and proliferation. Hr mediated NFκB regulation occurs during epidermal homeostasis but it is particularly important during the keratinocyte response to UVB irradiation where it is essential to the proper apoptosis of damaged keratinocytes. Thus, we postulate that Hr plays a crucial regulatory role in SCC development via inhibition of NFκB pathway after UVB irradiation. We conclude that Hr mutant mouse strains, including SKH-1, exhibit qualities associated with increased proliferation and cellular dysregulation, providing a molecular mechanism for their inherent tumor susceptibility.

Materials and Methods

Animals

SKH-1 mice were purchased from Charles River Laboratories (Kingston, NY). HRS/J (Hr−/−) were purchased from Jackson Laboratory (Bar Harbor, ME). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Columbia University (IACUC Protocol Number: AC-AAAA5875).

RNA and Protein Extraction

Dorsal skin was harvested from mutant and WT animals and divided into two parts; one portion was kept intact for histological analysis and RNA extraction (see below) and the other was used for epidermal isolation and subsequent RNA and protein extraction. Briefly, dorsal whole skin was scraped to remove excess subcutaneous fat and divided into small strips and incubated in 0.25% trypsin with EDTA (Invitrogen, Grand Island, NY) for 2 hrs at 37°C. The epidermis was mechanically separated from the dermis by scraping and incubated in SMEM with 10% FBS, 1% PS (Invitrogen) at room temperature with shaking for 30 min to separate the keratinocytes. Keratinocyte clumps were then filtered out using a 70 μm cell strainer (BD Biosciences, San Diego, CA) and the remaining single cell filtrate was pelleted by centrifugation. The cells were then resuspended in either RLT Buffer for RNA Extraction (Qiagen, Valencia, CA) or the Flag-Lysis Buffer (50 mM Tris-HCl pH 7.3, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 1 mM DTT, 10 % glycerol and fresh proteinase inhibitors) for protein extraction.

A portion of the whole skin was also homogenized in RLT Buffer and RNA was isolated from either the epidermis or the whole skin using an RNeasy Mini Kit (Qiagen) followed by DNase treatment. 2 μg of total RNA was used for the cDNA synthesis using Superscript III (Invitrogen) and a 2:1 ratio of random hexamers: oligo (dT) primers according to manufacturer’s instructions.

Protein was extracted from either epidermal extracts or whole tumors homogenized in Flag-Lysis buffer. Proteins were quantified using RD DC protein assay protocol as instructed by the manufacturer (BioRad, Hercules, CA). Relative band intensity was measured using ImageJ software.

Quantitative RT-PCR

Real time PCR was performed on a ABI 7300 (Applied Biosystems, Carlsbad, CA) using the Relative Quantification Assay Protocol Number: AC-AAAA5875).
and software. Primers (Invitrogen) were designed according to ABI guidelines. All were written 5’ to 3’:

mH ex 6 FTTCACATGTCGACTCAT
mH ex 6/7 RTGTTCAGG AAGACGA
mH ex 1.5 CTGTTGACGACAGCAGAAG
mH ex 16 FC TCTCCAGATGTTGTGC
m2M FATTGCAGGC CGCTTGATCTCA
m2M RTAAGAT GTGAGATATGTCCTCA
mTNF-α FGGCCTATGTCTCAGGCTT
mTNF-α RGGAGAAGTCCCTCCAGAT
mIL-1α FFACACCTGCTTGTTGTC
mIL-1α RTAGGTGTGATGATTACCAGTT
mMnSOD FGACCTGGCTTACGACTATGG
mMnSOD R GACCCTGCTTCCTTAATTGAAGC
mTRAIL FTCACCAAGGAGTAGAAGCAGC
mTRAIL RCTCACCCTTGCTTCCTGGAG

All reactions were performed using ABI Power Sybr Master Mix, 200nM primers and 200ng cDNA. The following protocol was used: step 1 95°C 2 min, step 2 95°C 10 min, step 3 95°C 15 sec, step 4 60°C 1 min, repeat step 2–4 for 40 cycles. All samples were run on a 1.5% agarose gel to confirm amplicon size. The data output was imported into the GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA), plotted, and the size was calculated. The Student’s T-test was performed using GraphPad Software (GraphPad Software, Inc., La Jolla, CA), and software. The p-values were calculated as standard error of the mean (SEM). Statistical analysis was performed between time points and samples as indicated using Student’s T-test.

UV irradiation protocol

For all protocols, irradiation was performed using eight FS72T12-UVB-HO lamps (Daavlin, Bryan, OH) and all animals were irradiated at 180 mJ/cm² UVB which has been established as approximately equivalent to 2 MEDs [30,31]. UVC was eliminated by a Kodac filter (TA401/407) (Kodak, Rochester, NY). The UVB dose was measured with a UVB Spectra 305 Dosimeter (Daavlin) and calibrated with a secondary radiometer (IL1700) (International Light, Peabody, MA).

Chronic UVB protocol. Female 9–11 week old HRS/J and shaved WT counterparts (n = 10 for each) were irradiated three times a week for a total of 50 weeks. Tumors were counted when they reached 2 mm in diameter on a weekly basis. After 25 and 30 weeks of irradiation mice were chosen for euthanization. At 42 weeks of irradiation mice were chosen for experimentation. Sections were done at 4 μm parallel to paravertebral line following standard procedures. Sections were treated with Antigen Unmasking Solution (Vector Labs, Burlingame, CA) then probed with phospho-1kB (ser32/36) (AbCam) followed by avidin-biotinylated HRP complex (ABC Elite system, Vector Labs). The antibody was detected with DAB and images were taken using an HRC Axiocam fitted onto an Axioplan2 fluorescent microscope.

Western Blots

Protein extracts were prepared as described above, while nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL). All samples were used for Western blotting analysis using standard protocols and the following primary antibodies: p65 (AbCam), p50 (Santa Cruz, Santa Cruz, CA), and p52 (Santa Cruz). Western blotting was done using standard protocols and the following primary antibodies: IkBα (Santa Cruz) IkBα (ser32/36) (AbCam), PCNA (Santa Cruz), Bcl-2 (Enzo Life Sciences, Farmingdale, NY), and p52 (Santa Cruz). Western blotting was done using standard protocols and the following primary antibodies: IκBα (Santa Cruz) IκBα (ser32/36) (AbCam), p50 (Santa Cruz), and p52 (Santa Cruz). Western blotting was done using standard protocols and the following primary antibodies: IκBα (Santa Cruz) IκBα (ser32/36) (AbCam), p50 (Santa Cruz), and p52 (Santa Cruz). Western blotting was done using standard protocols and the following primary antibodies: IκBα (Santa Cruz) IκBα (ser32/36) (AbCam), p50 (Santa Cruz), and p52 (Santa Cruz). Western blotting was done using standard protocols and the following primary antibodies: IκBα (Santa Cruz) IκBα (ser32/36) (AbCam), p50 (Santa Cruz), and p52 (Santa Cruz). Western blotting was done using standard protocols and the following primary antibodies: IκBα (Santa Cruz) IκBα (ser32/36) (AbCam), p50 (Santa Cruz), and p52 (Santa Cruz).

EMSA

3.5 pmol of NFκB consensus oligonucleotide (5’- AGTT-GAGGGAGTTTTCCGAGGC-3’) (Promega, San Louis Obispo, CA) was 5’ labeled with [γ-32P] ATP using T4 polynucleotide kinase (Promega). Nuclear protein extracts were incubated in Gel Shift Binding Buffer (Promega) containing 20% glycerol (v/v), 5mM MgCl₂, 2.5mM EDTA, 2.5mM DT, 250mM NaCl and 50mM Tris-HCl (pH 7.5) and labeled oligonucleotide. In competition experiments, a 100-fold molar excess of unlabeled probe was added before the labeled probe. Reaction products were separated on 4% polyacrylamide gel, after which the gel was dried and exposed to X-ray film overnight at -70°C.
Supporting Information

Figure S1 SKH-1 and Hr⁻/⁻ mice contain the same proviral insertion. A) Schematic layout of primer locations and insertion site. B) Using PCR primers designed to intron 5F and intron 6R1 (lane1) yields a product in all genotypes, while primers designed to intron9F, intron9R2 (lane2), and exon6/intron6F, intron6R3 (lane3) amplifies only in the WT samples indicating an insertion is present causing the PCR to fail. C) Using Hairless specific Hairless primer (ex6/intron6F) and viral specific reverse primers (lane3), products were observed only in the mutants indicating SKH-1 and Hr⁻/⁻ have the same viral insertion. D) DNA sequencing was performed to confirm the insertion as the same as that found in Hr⁻/⁻. Black arrow indicates the insertion site junctions. (TIF)

Figure S2 Hr⁻/⁻ animals are unaffected by chronic UVB irradiation until 30 weeks. A) At 22 weeks of chronic UVB irradiation, neither WT nor Hr⁻/⁻ animals show any signs of tumorigenesis. B) By 30 weeks of chronic UVB irradiation, Hr⁻/⁻ animals begin to develop small papillomas. (TIF)

Figure S3 PDTC treatment prevents tumorigenesis and activation of NFkB in SKH-1 animals. A) After 35 weeks of chronic UVB irradiation, SKH-1 animals given only tap water developed tumors with the gross morphology of aggressive SSCs. PDTC treated SKH-1 animals at the same time period develop significantly less tumors that appear to be less aggressive. B) EMSA using samples derived from unirradiated epidermis, non-tumor bearing epidermis from untreated and PDTC-treated animals, and tumors from untreated and PDTC-treated animals. PDTC treatment significantly decreases NFkB binding to target sequences (indicated by black arrow). C) Western blot of 1xβz and p-1xβz from unirradiated epidermis, non-tumor bearing epidermis from untreated and PDTC-treated animals, and tumors from untreated and PDTC-treated animals. PDTC treatment decreased p-1xβz expression in the non-tumor bearing skin of irradiated animals. (TIF)

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Author Contributions

Conceived and designed the experiments: HK AC AMC. Performed the experiments: HK AC CTL. Analyzed the data: HK AC CTL ALK DRB MA AMC. Contributed reagents/materials/analysis tools: DRB MA AMC. Wrote the paper: HK AC CTL.

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