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p38 MAP Kinase Plays a Functional Role in UVB-Induced Mouse Skin Carcinogenesis

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Abstract

UVB irradiation of epidermal keratinocytes results in the activation of the p38 MAPK pathway and subsequently activator protein-1 (AP-1) transcription factor activation and COX-2 expression. AP-1 and COX-2 have been shown to play functional roles in UVB-induced mouse skin carcinogenesis. In this study, the experimental approach was to express a dominant negative p38 α MAPK (p38DN) in the epidermis of SKH-1 hairless mice and assess UVB-induced AP-1 activation, COX-2 expression and the skin carcinogenesis response in these mice compared to wild-type littermates. We observed a significant inhibition of UVB-induced AP-1 activation and COX-2 expression in p38DN transgenic mice, leading to a significant reduction of UVB-induced tumor number and growth compared to wild-type littermates in a chronic UVB skin carcinogenesis model. A potential mechanism for this reduction in tumor number and growth rate is an inhibition of chronic epidermal proliferation, observed as reduced Ki-67 staining in p38DN mice compared to wild-type. Although we detected no difference in chronic apoptotic rates between transgenic and non-transgenic mice, analysis of acutely irradiated mice demonstrated that expression of the p38DN transgene significantly inhibited UVB-induced apoptosis of keratinocytes. These results counter the concerns that inhibition of p38 MAPK in a chronic situation could compromise the ability of the skin to eliminate potentially tumorigenic cells. Our data indicate that p38 MAPK is a good target for pharmacological intervention for UV induced skin cancer in patients with sun damaged skin, and suggest that inhibition of p38 signaling reduces skin carcinogenesis by inhibiting COX-2 expression and proliferation of UVB-irradiated cells.

Keywords

ultraviolet light; dominant negative p38; non-melanoma skin cancer; COX-2; AP-1

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Introduction

Mitogen activated protein kinase (MAPK) signaling is a known contributing factor to tumor promotion. Our laboratory and others have demonstrated a direct effect of ultraviolet light (UV)-induced MAPK activation in signaling events mediating skin carcinogenesis [1-4]. Skin tumor promotion is driven in part by the activation of the activator protein-1 (AP-1) transcription factor complex and the induction of pro-inflammatory genes, notably cyclooxygenase-2 (COX-2). UVA (320-400 nm) and UVB (280-320 nm) light induce AP-1 activity and COX-2 expression, and these events are both dependent on activation of MAPKs. Three of the four known families of MAPKs have been identified to play a role in cellular signaling cascades upstream of UVB-induced proliferation and survival responses in keratinocytes. Blocking p38 MAPK signaling has been shown to inhibit UVB-induced AP-1 activation in cultured keratinocytes and in mouse skin, specifically by reducing the phosphorylation of cyclic AMP response element binding protein (CREB) and inhibiting c-fos expression that drives AP-1 activity [1]. The goal of the current study was to directly link UVB-induced p38 MAPK activity to skin carcinogenesis and investigate the cellular response to chronic suppression of p38 MAPK activity induced by UVB irradiation.

Sunlight is the primary environmental carcinogen responsible for the high incidence of non-melanoma skin cancer (NMSC). In the U.S., NMSC accounts for 40% of all new cancers diagnosed, and the incidence is increasing as the population ages and ozone is depleted [5]. An estimated 96% of the 1 million new cases of skin cancer annually are NMSCs, including basal cell carcinomas and squamous cell carcinomas (SCCs) [5]. These lesions typically occur on the sun-exposed regions of the body, including the head, neck, face, arms and hands. The critical wavelengths of sunlight responsible for induction of skin cancer lie in the UV spectrum. The shorter wavelengths of the UVB range are particularly damaging in that UVB is a potent complete carcinogen capable of tumor cell initiation, promotion and progression. On the other hand, UVA is less effective as an initiating agent, but is still a critical factor in UV-induced tumor promotion and progression.

Mechanistically, UVB causes specific mutations in the tumor suppressor gene p53, and these initiated cells with p53 mutations have defective cell cycle checkpoint signaling and are resistant to apoptotic cell death [6-9]. Continued exposure to UVB drives clonal expansion of these initiated cells and ultimately gives rise to benign papillomas that can progress to invasive SCC. Our laboratory has demonstrated the important role that the AP-1 transcription factor complex plays in UV-induced skin tumor promotion [10]. We have demonstrated that UVB irradiation induces the expression of the c-fos proto-oncogene and that this is a major driver for AP-1 activity, as UVB-induced AP-1 consists primarily of JunD and c-Fos heterodimers [11]. The direct role of AP-1 in skin tumor development was also demonstrated through the use of a transgenic mouse (TAM67) that expresses a dominant negative c-Jun in the epidermis [10]. This dominant negative c-Jun inhibits UVB-induced AP-1 activation both in cultured keratinocytes and in mouse skin. After chronic treatment with UVB, the TAM67 mice developed 50% fewer skin tumors than their wild-type littermates and the total tumor burden of these mice was also significantly reduced.

Expression of c-Fos and ultimately AP-1 activation is known to be the result of upstream activation of the p38 MAPK and PI3-kinase signaling pathways. These pathways work in coordination to stimulate CREB activity. Activation of p38 MAPK mediates the phosphorylation of CREB at S133, while PI3-K acts upstream of Akt and GSK-3 β to dephosphorylate CREB at S129 and enhance its DNA binding. Activated p-CREB then binds to the cyclic-AMP response element (CRE) and an AP-1-like element, FAP-1, in the c-fos promoter to drive expression [12-14]. Pharmacologic inhibition of p38 MAPK with SB202190 by topical delivery in an SKH-1 AP-1-luciferase reporter mouse showed that

blocking p38 signaling could be an effective strategy for preventing UVB-induced AP-1 activation in mouse epidermis. This finding suggested that p38 was a chemopreventive target for prevention of skin cancer [15].

In addition to AP-1 activation, UVB-induced skin tumor promotion is characterized by increased eicosanoid formation and prostaglandin synthesis. This is regulated in part by UVB-driven expression of COX-2, a critical eicosanoid biosynthetic enzyme [4]. Importantly, the p38/CREB signaling pathway directly influences cox-2 transcription through a CRE within the promoter region of the gene similar to that found near c-fos. Our laboratory demonstrated that topical application of SB202190 prior to UVB treatment reduced COX-2 protein accumulation by 84% [1]. Targeting p38 MAPK could therefore impact UV-induced skin cancer by directly affecting AP-1-regulated gene expression, as well as COX-2 expression, and by suppressing the inflammation that results from excessive sun exposure by blocking the production of pro-inflammatory signaling intermediates.

The p38 MAPK family is comprised of 5 subtypes, p38 α , p38 β 1, p38 β 2, p38 γ , and p38 δ . p38 α and p38 β are widely expressed and their functions are indistinguishable in skin, as both are involved in the normal keratinocyte functions of differentiation, proliferation, and apoptosis. p38 δ also plays a role in keratinocyte differentiation, but p38 γ is not known to be expressed in skin (for review, see [16]). In the current study, our goal was to block p38 MAPK signaling through expression of a dominant-negative p38 α isoform (p38DN) in the epidermis of SKH-1 mice. We found through the utilization of a 25-week skin carcinogenesis protocol that p38DN mice developed significantly fewer tumors and tumors of smaller size compared to their wild-type littermates. We also determined that the rate of tumor growth was significantly reduced and tumor latency was significantly increased in the p38DN mice. We conclude that suppression of p38 MAPK results in decreased epidermal proliferation, in part through inhibition of AP-1 activity, and a reduction in COX-2 protein levels. Genetic alteration of the p38 signaling pathway resulting in suppression of UVB-induced skin tumor development confirms that p38 MAPK is a viable chemoprevention target.

Materials and Methods

Generation of p38 dominant negative transgenic SKH-1 mice

The K14MCS expression vector (a generous gift of Dr. Jeffery Arbeit, Washington University) used to generate the p38DN transgenic SKH-1 mice consists of a keratin K14 promoter upstream of a rabbit β -globin intron, a multiple cloning site, and an HGH poly adenosine sequence. K14MCS was linearized with BamHI and blunted with Klenow. Human p38 MAPK harboring T180A and Y182F point mutations at the Thr-Gly-Tyr activation site [17], was myc-tagged by PCR. The 1.2 kb myc-p38DN fragment was excised by digestion with BamHI and XbaI, and then blunted with Klenow. After gel purification and phenol-chloroform extraction of linearized DNA, myc-p38DN was ligated into K14MCS, and sequence and orientation were verified.

Generation of the p38DN mice was performed by the Genetically Engineered Mouse Model Shared Service at the University of Arizona. Four to five week old female SKH-1 mice (Charles River, Wilmington, MA) were super ovulated with standard methods. Briefly, mice were injected i.p. with 5 IU of progesterone (pregnant mare serum, National Hormone and Peptide Program) followed by i.p. injection of 5 IU human choriongonatropin (Sigma-Aldrich, St. Louis, MO) 46 hours apart. The females were placed with SKH-1 males immediately after HCG administration. The 0.5 d.p.c. zygotes underwent pronuclear injection and were implanted into the infundibulum of 0.5 d.p.c. pseudo pregnant Swiss Webster mice (Harlan Labs, Indianapolis, IN). The founder mice derived from injected

zygotes were identified using PCR with primers in the K14 promoter (K14 forward primer: 5'-AAGCAGTCGCATCCCTTTCC-3') and the 5' region of the p38DN transgene (p38DN reverse primer: 5'-ACAGGTTCTGGTATCGTTC-3'). Confirmation of p38DN protein expression was determined by Western analysis as described below. The line was expanded by breeding with commercial outbred SKH-1 mice.

Detection of p38DN transgene expression

Detection of the p38DN transgene in mouse epidermal tissue was performed by excising full thickness back skins immediately after sacrificing the mice and snap freezing the skins in liquid nitrogen. The epidermal portion of skin samples was separated from dermis by scraping frozen skins on dry ice with a scalpel. Scraped epidermis was pulverized using a liquid nitrogen chilled mortar and pestle under RNase-free conditions. For analysis of epidermal proteins, pulverized samples were lysed in buffer containing 1% Triton X-100, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 3 mM β-glycerophosphate, 1 mM NaVO₄, 10 mM NaF, and 1 mM PMSF. Epidermal samples were further processed by sonicating in 3×10s bursts. Insoluble materials were pelleted by centrifugation and lysates transferred to clean tubes. SDS-PAGE and Western analysis was performed as indicated below. For RNA purification, pulverized samples were treated according to the manufacturer's instructions for the Ambion ToTALLY RNA Kit (Applied Biosystems, Carlsbad, CA). cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was performed to detect p38DN transgene expression using the following primers: 5'-AAGCACTCGCATCCCTTTGC-3' and 5'-GCTTCTTAAGTCCACACGAT-3'.

UVB-induced skin carcinogenesis

A total of 13 female p38DN mice and 10 female wild-type littermates were used in a 25-week skin carcinogenesis study. The mice were housed at the Arizona Health Sciences Center in microisolator cages in accordance with the standards set by the Institutional Animal Care and Use Committee (IACUC) at the University of Arizona. Mice were irradiated three times weekly using a bank of 6 FS40 sunlamps (National Biological Corp., Beechwood, OH) with an initial dose of 0.9 kJ/m² UVB. This dose was increased each week by 25% to a final dose of 2.75 kJ/m² UVB, which was continued for the duration of the experiment. Skin tumors were counted and measured using digital calipers once weekly. Tumors that did not persist for a minimum of two consecutive weeks were excluded from analysis of tumor multiplicity. Tumor burden data consists of all tumors included in multiplicity analysis that were measured to be at least 1 mm diameter. At the end of the experiment, mice were sacrificed and back skins excised and immediately fixed in 10% formalin for subsequent IHC analysis.

Statistical analysis was performed by the Biometry Shared Service at the Arizona Cancer Center. One p38DN and one wild-type mouse died before the end of study, and these mice were excluded from analysis because each mouse developed a tumor greater in size than 10 times the average group burden. Descriptive statistics, including mean and standard deviation, were calculated for various outcome measures. Tumor count and tumor surface area at the end of study were compared between p38DN mice and wild-type mice using the nonparametric Kruskal-Wallis test. Linear mixed effect models were used to study the trajectories of tumor count and tumor surface area over time after appropriate transformation. Kaplan-Meier survival curves were plotted for the tumor-free proportion of mice in each genotypic group, and time to first tumor was compared between the two groups by log-rank test.

AP-1-luciferase activity in transgenic SKH-1 mice

Transgenic SKH-1 mice expressing an AP-1-luciferase reporter gene (AP-1-luc) have been described previously [10]. These mice were bred with p38DN mice to produce a double transgenic strain (p38DN/AP-1-luc). A total of 8 female p38DN/AP-1-luc mice and 12 female AP-1-luc control mice were utilized in an acute UVB-irradiation study and were housed according to IACUC regulations. Three 2 mm ear punch biopsies were collected from the right ears of these mice and immediately snap frozen in liquid nitrogen for subsequent analysis. On the following day, mice were irradiated with a single dose of 6 kJ/m² UVB. After 48 hours, mice were sacrificed and three 2 mm punch biopsies were collected from the left ears of each mouse and snap frozen. Ear punch biopsies were thawed on ice and lysed by mechanical homogenization in Passive Lysis Buffer included in the Luciferase Assay System kit (Promega, Madison, WI). Samples were centrifuged and cleared of insoluble material before performing a BCA protein assay (Pierce Biotechnology, Rockford, IL). A total of 10 µg protein/sample was used for the luciferase assay according to the manufacturer's instructions. Data are expressed as relative luciferase units ± SEM and statistical significance between the two groups was determined by Student's t test.

Immunohistochemical analysis of mouse skin

In chronically and acutely irradiated mice, full thickness back skins were collected from transgenic p38DN mice and wild-type littermates at the time of sacrifice. A portion of the collected skins were fixed in 10% neutral buffered formalin for 24 hours, processed and embedded in paraffin. Immunohistochemistry was performed by the Tissue Acquisition Cellular/Molecular Analytical Shared Service (TACMASS) at the University of Arizona. Routine hematoxylin and eosin stains were performed on three micron sections of tissue cut from the formalin fixed, paraffin embedded blocks. Immunohistochemistry was performed using antibodies directed against Ki-67 (Novocastra, Leica Microsystems, Bannockburn, IL) and cleaved caspase-3 (Cell Signaling Technologies, Danvers, MA). Tissue sections were stained on a Discovery XT Automated Immunostainer (Ventana Medical Systems). All steps were performed on this instrument using validated reagents, including deparaffinization, cell conditioning (antigen retrieval with a borate-EDTA buffer), primary antibody staining, detection and amplification using a biotinylated-streptavidin-HRP and DAB system and hematoxylin counterstaining. Following staining on the instrument, slides were dehydrated through graded alcohols to xylene and coverslipped with mounting medium. Images were captured using a Paxcam 3 camera with PAX-it Digital Image Management & Image Analysis and standardized for light intensity. No automated analysis of the data was performed. Blinded analysis of staining intensity was performed by a board certified histopathologist. For all staining, 5 fields per slide were analyzed at a magnification of 200x. Data are expressed as average number of stained cells/field, with 10 total fields for acute samples (2 mice per genotype) and 20 total fields for chronic samples (4 mice per genotype).

Western blotting for analysis of proteins in mouse skin

Back skin tissue collected from acutely irradiated mice (wild-type and transgenic) and from p38DN transgenic mice during initial characterization was immediately snap frozen in liquid nitrogen after sacrificing and then stored at -80°C for further processing. The epidermal portion was separated from the dermis as described above and sonicated. A Bio-Rad Dc protein assay was performed and 40 µg per sample were separated by SDS-PAGE. Proteins were transferred to PVDF membranes and the membranes were blocked for 1 hr in tris-buffered saline containing 0.1% Tween 20 (TBST) and either 2% BSA (for phospho-proteins) or 5% non-fat dry milk (for all others). Membranes were then incubated with primary antibodies diluted in the appropriate blocking buffer overnight at 4°C (c-myc, p38α, p-CREB, p-HSP27, p-JNK 1/2, p-ERK 1/2, ERK 1/2, Cell Signaling Technologies; COX-2, Santa Cruz Biotechnology, Santa Cruz, CA, α-tubulin, Calbiochem/EMD Chemicals,

Gibbstown, NJ). Membranes were washed with TBST, incubated with appropriate secondary antibodies, washed extensively and developed using Amersham ECL reagents (GE Healthcare Lifesciences, Piscataway, NJ). Densitometric analysis was performed using ImageJ software.

Results

Isolation and characterization of the p38 dominant negative transgenic mouse

To avoid repeated rounds of breeding later, the K14-myc-p38DN construct was injected into single-cell fertilized embryo from the SKH-1 strain, and carried forward on the SKH-1 line. After initial PCR-based genotyping of transgenic mice (Figure 1A), several mice were sacrificed in order to look for epidermal expression of the p38DN transgene. We were able to show expression of the transgene at both the mRNA and protein level (Figure 1B and 1C, respectively). The myc tag was identifiable using Western blots, and caused a shift in the protein weight which is also visible using a p38 antibody.

Expression of K14-p38DN transgene significantly inhibits UVB-induced skin tumorigenesis

Female p38DN transgenic mice (n=12) and their wild-type littermates (n=9) were exposed to a chronic UVB carcinogenesis protocol. The appearance, size and location of tumors were tracked throughout the duration of the experiment. As is shown in Figure 2A, expression of the p38DN transgene significantly inhibited the final tumor count from 9.3 tumors/mouse in the wild-type controls to 3.5 tumors/mouse in the transgenic mice, a 62.5% decrease ($p < 0.01$). The log rate of the increase in tumor number was also found to be significantly reduced ($p < 0.001$). In addition, the p38DN transgenic mice had a statistically significant ($p < 0.05$) lower overall tumor burden by 45.8% at the end of the study (5.9 mm² in wild-type versus 3.2 mm² in p38DN; Figure 2B), and the log rate of the tumor burden increase was significantly lower in the p38DN mice ($p < 0.001$). Tumor-free survival was significantly increased from 14 weeks in the wild-type mice to 17 weeks in the p38DN mice ($p < 0.05$; Figure 2C). The majority of the tumors were small lesions and papillomas; few squamous cell carcinomas were noted in either group. Therefore, expression of the p38DN transgene in the epidermis of SKH-1 mice significantly reduced UVB-induced tumor number and size.

Dominant negative p38 transgene expression inhibits markers of skin proliferation and apoptosis

After 25 weeks of UVB exposure, back skin samples from both wild-type and p38DN mice were fixed and stained for cleaved caspase-3 or Ki-67 expression. Only chronically exposed back skin, not tumors, were used for this analysis. Caspase-3 cleavage (a marker for apoptosis) was not significantly different between the two genotypes in the chronically-exposed samples (Figure 3A). However, Ki-67 staining (a marker for cellular proliferation) was reduced 29% in the p38DN samples compared to their wild-type counterparts ($p = 0.012$; Figure 3B). The response of back skin to acute UVB treatment was also compared between the groups using samples harvested 24hr after exposure. In this case, caspase-3 cleavage was inhibited 45% in the dominant negative mice when compared to wild-type ($p < 0.001$; Figure 3A). Ki-67 staining of acute samples also showed a trend towards inhibition in the dominant negative samples, although with marginal significance ($p = 0.098$; Figure 3B). These data provide evidence that UVB-induced proliferation and apoptotic responses are blunted in mice expressing dominant negative p38.

Dominant negative p38 transgene expression inhibits AP-1 activation in mouse skin compared to wild-type littermates after acute UVB exposure

Mice expressing the p38 dominant negative transgene were crossed with SKH-1 mice which ubiquitously express a luciferase reporter gene driven by four AP-1 binding sites, or TREs [18]. This “AP-1 luciferase” reporter is highly inducible with UVB exposure [10]. The UVB-induced luciferase response of the skin of female progeny harboring both the p38DN transgene and the AP-1 luciferase transgene were compared to female littermates harboring the luciferase transgene alone (Figure 4). Forty-eight hours after an acute dose of 6 kJ/m² UVB, ear punches from wild-type mice exhibited a 33-fold activation of AP-1 luciferase over pretreatment levels. This activation was reduced by 42% to a 13-fold activation in mice co-expressing the p38DN transgene ($p < 0.05$). We therefore have evidence that epidermal expression of the p38DN transgene results in inhibition of a significant player in UVB-induced skin carcinogenesis: the AP-1 transcription factor.

p38DN expression reduces UVB-induced COX-2 expression and does not affect epidermal thickness

Transgenic p38DN mice and wild-type littermates were irradiated with 6 kJ/m² UVB and sacrificed 48 hr later. Western analysis with band densitometry to assess COX-2 protein expression performed on epidermal lysates prepared from mouse back skins revealed that expression of the p38DN transgene significantly reduced COX-2 protein levels in UVB-irradiated mice (Figure 5A). In untreated mice, COX-2 expression was not significantly lower in the dorsal epidermis of p38DN mice compared to wild-type controls (Figure 5B). COX-2 expression was induced by UVB at a similar level in both p38DN mice (4.7 fold increase) and wild-type mice (4.2 fold increase) after 48 hr, but the total COX-2 protein levels were significantly reduced 22.8% by expression of the p38DN transgene ($p < 0.01$). These data indicate that inhibition of the p38 MAPK pathway significantly reduces COX-2 expression and suggest that chronic reduction of COX-2 levels in the skin could be a contributing factor to the inhibitory effect of p38DN on skin cancer development.

Because p38 has been implicated in keratinocyte differentiation, we also evaluated the effects of p38DN expression on epidermal thickness and skin morphology by IHC in the mock irradiated mice. We observed no measurable differences across multiple skin sections from p38DN and wild-type mice (data not shown), indicating that expression of dominant negative p38 α does not significantly affect keratinocyte differentiation, nor does it cause observable morphological changes. This finding suggests that p38DN expression is not significantly affecting the normal functions of endogenous p38 α , p38 β , or p38 δ in mouse skin and also suggests that the principal effect of the p38DN transgene is inhibition of the cellular events induced by UVB.

Expression of dominant negative p38 inhibits activation of downstream signaling components in mouse epidermis

Transgenic p38DN mice and wild-type controls were assessed for UVB-induced acute activation of the p38 MAPK signaling pathway. Mice were exposed to 6 kJ/m² UVB and then sacrificed one hr later. Western analysis of isolated epidermal lysates was performed to demonstrate expression of the p38DN protein and the effect of p38DN expression on UVB-induced CREB and HSP27 phosphorylation (Figure 6). Expression of p38DN protein varies between mice, but clear expression of the transgene is evident when compared to wild-type littermates by the presence of a band that migrates slightly slower than endogenous p38 α . The data clearly indicate a reduction in both UVB-induced phospho-CREB and phospho-HSP27 in p38DN mice compared to wild-type. Densitometric analysis of UVB-induced phospho-CREB levels normalized to α -tubulin were found to be significantly reduced in the p38DN mice compared to wild-type, with $p = 0.031$. The reduction of HSP27

phosphorylation in the p38DN group did approach statistical significance, with $p = 0.056$. Western analysis was also performed for phospho-ERK 1/2 and phospho-JNK 1/2 to determine whether expression of p38DN affects UVB-induced phosphorylation of these two MAPK family members. Densitometric analysis of α -tubulin-normalized phospho-JNK levels revealed no difference between the wild-type and p38DN groups ($p = 0.458$). Similar to JNK, UVB-induced ERK 1/2 phosphorylation normalized to α -tubulin was not significantly different between the two groups ($p = 0.436$). We conclude from these data that the principal effect of p38DN expression is attenuation of UVB-induced p38 MAPK signaling and that this contributes significantly to the inhibition of skin carcinogenesis in the chronic UVB model.

Discussion

UVB irradiation is an established etiologic factor in the development of SCC [19]. The development of UVB-induced SCC occurs as a result of changes at both genetic and epigenetic levels. Genetically, UVB induced DNA damage is a critical event in the initiation of SCC [20,21]. In contrast skin tumor promotion and progression are often dependent upon changes in gene expression due to epigenetic events.

UVB induction of AP-1 and COX-2 has clearly been associated with SCC development [22,23]. We have shown that the expression of a dominant-negative c-jun (TAM67) in the epidermis of hairless SKH-1 mice inhibits UVB induction of SCC. Knockout of the COX-2 gene and inhibition of COX-2 activity have been shown to inhibit UVB skin carcinogenesis in the mouse [23,24]. We have provided evidence that p38 MAPK plays an important role in increasing both AP-1 and COX-2 activity in the human keratinocyte cell line HaCaT [3,22]. We have recently published data using a pharmacologic inhibitor of p38 MAPK to show the involvement of this kinase in UVB induced activation of AP-1 and COX-2 expression in the SKH-1 mouse epidermis [1]. In this present work we have used a genetic approach to inhibit UVB induced p38 MAPK activity in the epidermis of the mouse and have shown that this inhibition results in a significant reduction of UVB induced mouse skin carcinogenesis.

We observed that expression of the p38DN transgene significantly inhibited both the average tumors per mouse and the average tumor burden. In addition, the length of tumor-free survival was increased in mice expressing p38DN. These results were predicted based on our published findings that pharmacologic inhibition of acute UVB induced p38 MAPK in the epidermis of SKH-1 mice inhibited the induction of AP-1 activity and COX-2 expression. Since AP-1 and COX-2 have been shown to play functional roles in UVB-induced skin carcinogenesis, we hypothesized that inhibition of signaling events leading to their activation and expression would inhibit skin carcinogenesis. Hildesheim, et al. [25] demonstrated in acutely UVB irradiated SKH-1 mouse epidermis that i.p. treatment with the p38 MAPK inhibitor, SB202190, protected against several detrimental effects of UV irradiation. These effects included reductions in sunburn cell formation/apoptosis, inflammation and a hyperproliferative response. The authors speculate that selectively blocking p38 activation with an inhibitor could prove beneficial in treating victims with severe sunburn known to trigger the p38 MAPK pathway. However, these authors also caution that the use of selective p38 MAPK inhibitors in treatment of chronic repeated sun damage may not only compromise the ability of the skin to monitor the genomic integrity of their cells but also their ability to eliminate potentially tumorigenic cells. Our results using a genetic approach to inhibit chronic UVB induced p38 MAPK signaling suggest that UVB induced skin carcinogenesis is not enhanced but instead inhibited. We found in the p38DN mice that after acute exposure to UVB there was a significant reduction in epidermal apoptosis. These results are in agreement with those found by Hildesheim, et al. [25] using a pharmacologic inhibitor of p38 MAPK. In contrast to acute UVB exposure we found with

chronic UVB exposure the mice expressing the p38DN transgene demonstrated no significant difference in epidermal apoptosis compared to the non-transgenic littermates. Our results counter the concerns expressed by Hildesheim, et al. [25] that inhibition of p38 MAPK in a chronic situation may compromise the ability of the skin to eliminate potentially tumorigenic cells. Instead, we found in the chronically UVB exposed p38DN transgenic mice there was a significant reduction in the number of proliferative cells. This inhibition of UVB induced proliferation by the p38DN transgene could explain, in part, the inhibition of skin tumor promotion and tumor growth.

In another published study [26] it was shown that blockade of the p38 MAPK pathway could offer an effective approach to reducing or preventing skin damage resulting from acute solar radiation. Kim, et al. found that oral administration of the p38 inhibitor, SB242235, prior to UVB irradiation of SKH-1 mice blocked activation of the p38 MAPK cascade resulting in inhibition of IL-6, KC (murine IL-8) and COX-2 expression. These authors did not study the effects of inhibiting p38 MAPK in a chronic model of UVB irradiation of the skin; however, it is known that chronic inflammation plays a functional role in skin tumor promotion. Therefore, since we demonstrated a significant reduction in COX-2 protein levels after UVB irradiation in the p38DN mice, chronic inhibition of p38 MAPK activation could have resulted in inhibition of inflammation and skin tumor promotion induced by UVB irradiation.

The results of this study using a genetic approach to inhibit p38 MAPK signaling in the epidermis of chronically UVB irradiated mice has shown that this inhibition in signaling results in a reduction in number of skin tumors and a reduction in tumor growth. The mechanism appears to involve a reduction in chronic hyperproliferation, in part due to inhibition of CREB, HSP27, and AP-1 activation, induced by repeated UVB irradiation of the skin. Though there was an inhibitory effect of the transgene on acute UVB induced apoptosis in the epidermis, there was no effect of transgene on apoptosis in the chronically irradiated epidermis. We also provided evidence that the reduction in skin tumor development could be mediated in part through suppression COX-2 expression. In conclusion, p38 MAPK activation plays a functional role in UVB induced mouse skin carcinogenesis and is a potential target for pharmacological intervention in UVB induced skin cancer development.

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Abbreviations

AP-1	activator protein-1
BCA	bicinchoninic acid
COX-2	cyclooxygenase-2
CRE/CREB	cyclic AMP response element/CRE binding protein
GSK	glycogen synthase kinase
HGH	human growth hormone
MAPK	mitogen activated protein kinase
NMSC	non-melanoma skin cancer
p38DN	p38 dominant negative
RT-PCR	reverse transcriptase-polymerase chain reaction
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
TBS	tris-buffered saline
TRE	TPA (12-O-tetradecanoylphorbol-13-acetate) response element
UV	ultraviolet

Figure 1. Detection of p38DN expression in the epidermis of transgenic SKH-1 mice
SKH-1 mice genotyped as p38DN positive were sacrificed and back skins were harvested and immediately snap frozen. Epidermal RNA and protein was extracted as indicated in *Materials and Methods* and subjected to RT-PCR and Western analysis, respectively. (A) A sample of genotyping from tail DNA shows a 1kb PCR product generated using K14 forward primers and a p38DN reverse primers spanning a rabbit β -globin intron as described in *Materials and Methods*. (B) PCR was performed on extracted RNA without first performing a DNase reaction for the sole purpose of preserving the extracted genomic DNA. PCR generated a single band at 688 bp in samples not first treated with RT (Lane 1), consistent with the predicted PCR product from genomic DNA. In RT treated samples (Lane 2), an additional band at 302 bp is visible, which was generated from cDNA after splicing of the rabbit β -globin intron within the expressed RNA. (C) Western blot analysis of isolated protein revealed p38DN protein expression in mouse epidermis using anti-myc and anti-p38

antibodies. PCR and Western data are representative samples of transgenic p38DN mice and/or wild-type littermates.

Figure 2. Expression of p38DN reduces UVB-induced skin tumorigenesis

A 25-week mouse skin carcinogenesis experiment was performed using female p38DN transgenic SKH-1 mice (n=12) and female wild-type littermates (n=9) as indicated in *Materials and Methods*. (A) Tumor multiplicity data consist of the average weekly tumor numbers per group for all recorded skin tumors from the point at which they were first observed. Tumors had to be present for at least two consecutive weeks to be counted and therefore included in the multiplicity data. Significant differences were found between the groups for tumor number at the end of the experiment ($p<0.01$) as well as for rate of tumor development throughout the experiment ($p<0.001$). Data are expressed as mean tumors/mouse $\pm$ SEM. (B) Tumor burden data consist of measured circular surface areas for all tumors greater than 1 mm diameter. Statistical analysis revealed significant differences in tumor burden at the end of the experiment ($p<0.05$), as well as in the rate of tumor growth between the two groups ($p<0.001$). Data are expressed as mean tumor burden $\pm$ SEM. (C) Tumor incidence data for both groups are represented using a Kaplan-Meier plot of tumor-

free survival. Time to first tumor was analyzed for both groups, and a significant difference ($p < 0.05$) was found between the groups indicating that tumor-free survival was longer in the p38DN group (- - -) than in the wild-type group (—).

Figure 3. p38DN expression blocks proliferation of basal keratinocytes and caspase-3 cleavage in irradiated mouse skin

Skin isolated from mice at the end of the 25-week skin carcinogenesis study and skin from mice irradiated acutely (24 hr post-UVB) with 6 kJ/m² UVB was analyzed by IHC for caspase-3 cleavage and expression of Ki-67 to assess apoptosis and proliferation, respectively. (A) Differences in caspase-3 cleavage were observed only in the acutely irradiated mice. Quantification and analysis of staining indicated a significant difference between the p38DN and wild-type groups (*, $p < 0.05$). (B) Ki-67 staining was evident in both chronically and acutely irradiated mice. Quantification and analysis of staining indicated a significant difference between p38DN and wild-type groups in the chronically irradiated mice only (*, $p < 0.05$). Data are expressed as average cell counts/field $\pm$ SEM.

Figure 4. p38DN expression reduces AP-1 activity in mouse skin

Transgenic AP-1-luciferase reporter mice were crossbred with p38DN mice to produce double transgenic mice. Right ear punch biopsies were first collected from female double transgenic mice (n=8) and their AP-1-luciferase/p38 wild-type female littermates (n=12) prior to irradiation with 6 kJ/m² UVB. The mice were sacrificed 48 hr later, and left ear punch biopsies were collected for analysis of luciferase expression. There is a statistically significant reduction in AP-1-luciferase activity in the p38DN mice compared to the wild-type littermates (*, p<0.05). Data are expressed as mean relative luciferase units/10 µg protein ± SEM, and significance was determined using Student's t test.

Figure 5. p38DN expression reduces COX-2 protein levels in acutely UVB-irradiated mouse skin Female SKH-1 mice expressing p38DN and wild-type SKH-1 littermates (each group, n=5) were irradiated with 6 kJ/m² UVB and sacrificed 48 hr later. Epidermal proteins were isolated and Western analysis performed as indicated in *Materials and Methods*. (A) Western blotting shows COX-2 expression in mock-irradiated mice (wild-type, n=2; p38DN, n=3; Lanes 1-5) and in UVB-irradiated p38DN mice (Lanes 6-10) and wild-type littermates (Lanes 11-15). (B) Densitometry using ImageJ software demonstrated no significant reduction in basal COX-2 levels in mock irradiated p38DN mice compared to wild-type, but a statistically significant 22.8% reduction in UVB-induced COX-2 levels in UVB irradiated p38DN mice (*, p<0.01). Data are expressed as mean relative densitometric units $\pm$ SEM, and significance was determined using Student's t test.

Figure 6. Expression of p38DN suppresses UVB-induced p38 signaling

Female SKH-1 mice expressing p38DN and wild-type littermates were sacrificed 1 hr after exposure to 6 kJ/m² UVB. Epidermal proteins were isolated as indicated in *Materials and Methods*. Western blotting shows expression of p38DN protein as a slower migrating band than endogenous p38. Inhibition of UVB-induced CREB and HSP27 phosphorylation by p38DN was confirmed by densitometry ($p = 0.031$ and 0.056 , respectively). There was no effect of p38DN expression on the levels of phospho-JNK 1/2 and phospho-ERK 1/2. Lanes 1, 2: WT mock irradiated mice; lanes 3, 4: p38DN mock irradiated mice; lanes 5-8: WT 6 kJ/m² UVB irradiated mice; lanes 9-12: p38DN 6 kJ/m² UVB irradiated mice.