

Ultraviolet Radiation Stimulates Expression of Snail Family Transcription Factors in Keratinocytes

Laurie G. Hudson,¹ Changsun Choi,² Kimberly M. Newkirk,² Jaipriya Parkhani,¹ Karen L. Cooper,¹ Ping Lu,³ and Donna F. Kusewitt^{2*}

¹College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

²Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio

³Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio

The related zinc finger transcription factors Slug and Snail modulate epithelial mesenchymal transformation (EMT), the conversion of sessile epithelial cells into migratory fibroblast-like cells. EMT occurs during development, wound healing, and tumor progression. Growth factors, acting through mitogen-activated protein kinase (MAPK) cascades, regulate expression of Slug and Snail. Expression of Snail family transcription factors appears to be elevated in UVR-induced murine squamous cell carcinomas (SCC). We report here that ultraviolet radiation (UVR), which activates MAPK cascades, also stimulates Snail and Slug expression in epidermal keratinocytes. UVR exposure transiently elevated Slug and Snail mRNA expression in human keratinocytes *in vitro* and mouse epidermis *in vivo*. This induction was mediated, at least in part, through the ERK and p38 MAPK cascades, as pharmacological inhibition of these cascades partially or completely blocked Slug and Snail induction by UVR. On the other hand, UVR induction of Slug and Snail was enhanced by inhibition of JNK. Slug appears to play a functional role in the acute response of keratinocytes to UVR, as UVR induction of keratin 6 in the epidermis of Slug knockout mice was markedly delayed compared to wild-type mice. Slug and Snail are known to regulate molecules important in the cytoskeleton, intercellular adhesion, cell motility, and apoptosis, thus it seems probable that transiently or persistently elevated expression of these factors fosters the progression of UVR-induced SCC. © 2007 Wiley-Liss, Inc.

Key words: skin; keratinocyte; ultraviolet rays; snail family transcription factors

INTRODUCTION

Ultraviolet radiation (UVR) is the primary etiologic agent for nonmelanoma skin cancer in humans [1–4]. Studies in murine models of nonmelanoma skin cancer, specifically squamous cell carcinoma (SCC), have shown conclusively that both UVR-induced genetic changes (particularly mutational inactivation of the p53 tumor suppressor gene) and epigenetic changes in gene expression (notably enhanced expression of components of the AP-1 transcription factor) contribute to skin cancer development [reviewed in 2,5].

Some UVR-induced SCC in mice evolve into aggressive spindle cell carcinomas that resemble fibrosarcomas [6–9]. Morphologic changes occurring during this transition include attenuation of desmosomes and adherens junctions and loss of cytokeratin expression [6–9]. This process resembles the epithelial mesenchymal transformation (EMT) that takes place in the embryo [10,11]. During embryonic development, the Snail family transcription factors Snail and Slug drive EMT [reviewed in Reference 12]. Recent findings demonstrate that Snail and Slug also play an important role in EMT-like events occurring in adult skin, including wound

healing and SCC progression [13,14]. Slug expression is transiently increased in migrating keratinocytes at the margins of healing cutaneous wounds, a site of EMT-like changes in the epidermis [14,15]. In chemically induced murine SCC, Snail and Slug are expressed in cell lines derived from the tumors and levels of Snail expression correlate with tumor stage [13]. Moreover, demethylation of the Snail promoter and enhanced Snail expression is associated with the transition from epithelial to spindle cell phenotype in such tumors [16]. Querying the Global Gene Expression Group SAGE profile of murine SCC

Abbreviations: UVR, ultraviolet radiation; SCC, squamous cell carcinoma; EMT, epithelial mesenchymal transformation; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; MED, minimal erythral dose; RT-PCR, reverse transcription-polymerase chain reaction; EGF, epidermal growth factor; FGF-1, fibroblast growth factor-1.

*Correspondence to: Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Road, Columbus, OH 43210.

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(<http://sciencepark.mdanderson.org/ggeg/default.html>) compiled at the University of Texas Science Park [17] reveals that tags unique for Slug were 3–9 times more frequent in SCC than in normal epidermis. Together, these findings highlight an emerging role for Snail family transcription factors in cutaneous biology.

A variety of Snail family transcriptional targets have been identified that may modulate EMT-like events during tumor progression, particularly genes encoding adhesion molecules and associated cytoskeletal elements important in cell attachment and motility [reviewed in References 18–20]. Both Slug and Snail repress expression of E-cadherin, an important component of adherens junctions, by binding to E-box-like promoter sequences [21–24]. Additionally, these transcription factors modulate expression of claudin, a constituent of tight junctions, and plakophilin, a component of desmosomes [19,25]. A variety of intermediate filaments and microfilaments associate with cell:cell and cell:substrate adhesion structures to stabilize cell structure and promote movement across a substrate. Slug and Snail have been shown to regulate, either directly or indirectly, expression of several of these cytoskeletal elements, including cytokeratins important in structural stability and actin binding proteins that control cell motility [19,26,27].

Ras-dependent stimulation of mitogen-activated protein kinase (MAPK) pathways is essential for full induction of EMT in adult cells, and enhanced transcription of Snail family members plays a central role in MAPK-dependent EMT [18,28,29]. UVR also stimulates signaling through MAPK pathways [reviewed in Reference 30], although induction of Snail family members by UVR has not previously been reported. Based on the role of Slug and Snail in EMT-like events in tumors, the importance of MAPK pathways in Slug and Snail induction, and the ability of UVR to activate MAPK cascades, we investigated the ability of UVR to induce expression of Slug and Snail in the epidermis via MAPK pathways.

In the present studies we show that UVR induces transient expression of Snail and Slug in cultured human keratinocytes *in vitro* and in mouse skin *in vivo*. UVR-stimulated Snail and Slug expression is driven, at least in part, via MAPK signaling pathways. In the absence of Slug, UVR induction of keratin 6, a marker of keratinocyte proliferation and altered differentiation [31,32], is markedly delayed, indicating a functional role for Slug in the acute cutaneous response to UVR. These results suggest involvement of Snail family transcription early in UVR-induced skin carcinogenesis and establish a link between UVR-induced alterations in cellular signaling cascades and expression of transcription factors implicated in EMT-like events taking place during SCC progression.

MATERIALS AND METHODS

UVR Source

UVR was obtained from banks of Philips TL20 W/12 RS UVB lamps (American Ultraviolet Company, Murray Hill, NJ). These lights emit wavelengths of light between 280 and 400 nm, with a peak at 313 nm. The lamps emit approximately 60% UVB wavelengths and 40% UVA wavelengths, with less than 3% of the radiation in the UVC range [33]. Radiation was either unfiltered or filtered through Kodacel (Eastman Kodak, Rochester, NY), which removes all UVR wavelengths below 290 nm [34,35].

Cell Culture Studies

The SCC 12F cell line was employed for studies examining UVR induction of Slug and Snail mRNA. This cell line was derived from a well-differentiated human SCC arising on facial epidermis and was kindly provided to us by Dr. William A. Toscano, Jr. (University of Minnesota, Minneapolis, MN). SCC 12F cells maintain a characteristic keratinocyte morphology, undergo density arrest, and are non-tumorigenic in nude mice [36–38]. Cells were grown in 60-mm culture dishes in DMEM:F12 medium consisting of 50% Dulbecco's modified Eagle's medium and 50% Hams-F12 medium (Sigma, St. Louis, MO) containing the antibiotics penicillin and streptomycin, 2 mM of L-glutamine, and 5% of fetal bovine serum (Gibco, Gaithersburg, MD). To minimize basal expression of Slug and Snail, all studies were performed on completely confluent cells maintained for 2 d in serum-free medium. Immediately before UVR exposure, culture medium was removed and reserved, cells were rinsed with phosphate-buffered saline (PBS) and exposed in PBS to UVR from Kodacel-filtered or unfiltered lamps. After exposure, the reserved serum-free medium was replaced.

To determine if Slug and Snail induction by UVR was dose-dependent *in vitro*, cells were exposed to 0, 150, 300, 600, or 1200 J/m² of filtered or unfiltered UVR and harvested 2 h later. Viability studies performed with the CellTiter 96 AQueous Cell Proliferation kit from Promega (Madison, WI) as directed demonstrated that, at 2 h postexposure, the viability of cells exposed to all doses of UVR from 150 to 1200 J/m² was more than 50% that of unexposed cells (data not shown). To examine the time course of Slug and Snail induction by UVR, cells were exposed to 300 J/m² of unfiltered UVR and harvested 0.5, 1, 2, 4, 24, or 48 h later. The viability of cells exposed to a dose of 300 J/m² UVR was measured as described above at various times after exposure. Viability declined to a low of approximately 50% of the viability of unexposed cells at 6 h postexposure, then rebounded to approach the level of unexposed cells by 24 h after exposure (data not shown).

Pathways involved in UVR induction of Slug and Snail were identified by pretreating cells with 10 μ M MAPK inhibitor for 2 h before UVR exposure, exposing cells to 300 J/m² UVR from unfiltered lamps, and harvesting cells 2 h after UVR exposure. As for other studies, cells were exposed in PBS and reserved medium, containing inhibitor, was replaced after exposure. This UVR dose falls within the lower range of UV-B doses employed in previous studies of MAPK activation in vitro (100–400 J/m²) and well below maximal doses of UV-B sometimes employed in such studies (4000–8000 J/m²) [reviewed in Reference 30]. MAPK inhibitors were obtained from Calbiochem (San Diego, CA) and included those that block MEK (U0126), JNK (SP600125), and p38 (SB202190) activity. These inhibitors are widely employed in cultured cells to dissect the contribution of signaling through different MAPK pathways to a variety of endpoints; the inhibitors were selected to allow interruption of signaling through each of the three major MAPK pathways. Additional studies to determine the contribution of UVC to Slug and Snail induction were carried out by exposing cells to 300 J/m² from Kodacel-filtered or unfiltered lamps and harvesting the cells 2 h after exposure.

Animal Studies

Studies in outbred hairless mice were performed to confirm UVR induction of Snail and Slug in vivo. Young adult SKH-1 mice (Charles River, Wilmington, MA) were exposed to 0, 1.5, or 3.0 minimal erythral doses (MED) of UVR from Kodacel-filtered Philips lamps (4800 J/m²). A value of 2240 J/m² per MED was based on previous studies employing the same strain of mice and the same light source [39]. Four mice were sacrificed by carbon dioxide inhalation 2, 6, 24, 48, and 96 h after exposure to 1.5 MED. One set of four mice was not exposed to UVR; one set of four mice was exposed to three MED and skin was harvested 24 h after exposure. After sacrifice, dorsal skin was removed and rapidly frozen in liquid nitrogen for storage at –80°C.

To examine the role of Slug in the cutaneous response to UVR, 18 Slug knockout mice and 18 of their wild-type littermates, all on an inbred 129 background [40], were shaved then completely depilated by Nair treatment. Two days later, 12 of the mice were exposed to 3 MED (4800 J/m² UVR) from Kodacel-filtered Philips lamps. Three mice of each genotype mice were killed by CO₂ inhalation at 12, 24, 48, 72, and 96 h after exposure. Shaved and depilated knockout and wild-type mice (3 per genotype) served as unexposed controls. Immediately after death, dorsal skin was removed and fixed in 10% neutral buffered formalin for subsequent paraffin embedding and sectioning. After antigen retrieval (TRS, Dako, Carpinteria, CA), staining for keratin 6 was carried out with a rabbit anti-keratin 6 primary antibody (Covance, Berkeley, CA) at a

dilution of 1:500, a biotinylated goat anti-rabbit secondary antibody (Vector, Burlingame, CA), the Vector ABC Elite kit with diaminobenzidine as chromagen, and a hematoxylin counterstain.

RNA Isolation and cDNA Synthesis

To obtain keratinocyte RNA from SKH-1 mouse skin, the epidermis was vigorously scraped from frozen skin samples, with a sterile scalpel blade, and placed immediately in Trizol (Invitrogen, Carlsbad, CA). Remaining dermis was fixed in formalin and processed routinely for histopathology to confirm that all epithelium had been removed (data not shown). Total RNA from human cells and mouse epidermis was isolated with Trizol as directed by the manufacturer (Invitrogen). DNA was removed with the DNA-free kit (Ambion, Austin, TX) as directed, and cDNA was produced from 500 ng of RNA with SuperScript II reverse transcriptase and oligo(dT) primers as recommended by the enzyme supplier (Invitrogen).

Quantitative mRNA Measurement

For cDNA from cultured human cells, quantitative polymerase chain reaction (Q-PCR) was performed with the Brilliant SYBR Green QPCR mix (Stratagene, LaJolla, CA) as directed and 100 nM of each primer. Primer sets included those for human Slug (5'-CCC-TGAAGATGCATATTCGGAC-3'; 5'-CTTCTCCCCC-GTGTGAGTTCTA-3'), Snail (5'-CGGAAGCCTAACT-ACAGCGA-3'; 5'-GGACAGAGTCCCAGATGAGC-3'), and glyceraldehyde 3-phosphate dehydrogenase GAPDH (5'-GCCGTGGAATTTGCCGT-3'; 5'-GCC-ATCAATGACCCCAT-3'). Amplifications were carried out on a Stratagene MX3000P Real-Time PCR System. Forty-five cycles of 94°C (30 s), 60°C (30 s), and 72°C (30 s) were performed. Electrophoresis of the products revealed single bands of the appropriate size (data not shown). RNA concentrations were calculated with the LinReg PCR program [41] and normalized to GAPDH values. Our preliminary studies showed that UVR exposure did not significantly alter levels of GAPDH expression in 12F SCC cells (data not shown), indicating that GAPDH was an appropriate internal control for this quantitation. For estimates of absolute copy number, Slug and Snail standards were obtained by PCR amplification of the genes from human keratinocyte DNA. The PCR products were purified and inserted into the pCRII-TOPO vector by standard techniques (Invitrogen). Serial dilutions of the pCRII-TOPO-Slug and Snail vectors were used in the quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis to obtain a standard curve. Copy number of experimental samples was determined from this standard curve [42].

Quantitative RT-PCR on mouse samples was carried out as described above, with mouse Slug (5'-GATGTGCCCTCAGGTTTGAT-3'; 5'-ACACATT-

GCCTTGTGTCTGC-3') and Snail (5'-TGTCAGAGGCTACACCTCA-3'; 5'-CTCACTGCCAGGACTCCTTC-3') primers. Primers for GAPDH (5'-ACCCAAGACTGTGGATGG-3'; 5'-CACATTGGGGGTAGGAACAC-3') were also used; electrophoresis of the products revealed single bands of the appropriate size (data not shown). However, UVR clearly induced GAPDH expression in mouse skin (data not shown), therefore, Slug and Snail standards were produced and employed for quantification as described above.

Western Blotting for Slug and Snail

UVR-exposed and control cells were rinsed with ice-cold PBS and protein was harvested in MAPK collection buffer (62.5 mM of Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM of dithiothreitol, and 0.1% w/v bromophenol blue). Cell lysates were clarified at 10 000g (4°C, 10 min) and 20 µL of total cell lysate resolved by SDS-PAGE. For Slug and Snail immunodetection, proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). For Slug detection, membranes were blocked in a 1:10 dilution of BlokHen (Aves Labs, Tigard, OR) in PBS containing 0.05% Tween-20 and probed with an affinity-purified polyclonal chicken antibody (1:4000) raised against a keyhole limpet hemocyanin-conjugated synthetic peptide representing a small portion of the murine Slug protein (CZEERLQPKLSDPHAIEAEK) not shared with Snail (Aves Labs). Detection was carried out with a horseradish peroxidase-labeled goat antibody raised against chicken immunoglobulin (Aves Labs) at a dilution of 1:5000. Westerns to detect Snail were blocked in 5% nonfat dry milk in TBST and probed with anti-snail antibodies (E-18 and T-18 at 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) as recommended by the antibody supplier. For both Slug and Snail Western blots, membranes were developed with the SuperSignal chemiluminescent detection system (Pierce, Rockford, IL). The original membranes were stripped and reprobed with antibodies against β -tubulin (H-235, Santa Cruz Biotechnology) to insure equal protein loading.

MAPK Activation Studies

For studies of MAPK activation, SCC 12F cells were grown to approximately 60% confluence in 60-mm plates, then placed in serum-free medium consisting of DMEM:F12, 0.1% bovine serum albumin, 2 mM of L-glutamine, 500 U penicillin/mL, 0.05 mg/mL of streptomycin overnight. Cells in PBS were exposed to 300 or 600 J/m² UVR at room temperature then incubated at 37°C for 0.5, 1, or 2 h. Anisomycin (10 ng/mL) or epidermal growth factor (EGF) (20 nM) was added to one plate per treatment set as a positive control for p38 and JNK or ERK activation, respectively. Negative controls were untreated plates

subjected to identical manipulations without UVR exposure. At collection, control and treated cells were washed with ice-cold PBS and harvested, clarified, and separated as described above. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA) and probed with anti-phospho-JNK, anti-phospho-ERK, or anti-phospho-p38 antibodies (Cell Signaling, Beverly, MA) according to the vendor's instructions. The membranes were developed with the SuperSignal chemiluminescent detection system (Pierce). The original membranes were stripped and reprobed with an anti-total p38 antibody (Cell Signaling Technology) to insure equal protein loading.

Statistical Analysis

Each in vitro experiment included 1–3 replicate samples for each treatment; each experiment was repeated 2–4 separate times. In all cases, the value obtained for each treated sample was standardized first to the GAPDH levels for that sample then to basal levels of Slug and Snail for that experiment. Results for different treatment groups were compared by the one-tailed Student *t*-test for paired samples. For in vivo studies with SKH-1 mice, four mice were examined at each time point after UVR exposure. Quantitative RT-PCR for each mouse sample was performed in triplicate, and the values were averaged. Levels of expression were determined by comparison to a standard curve generated with cloned copy number standards [42]; these values were then indexed to basal levels of Slug and Snail expression. Results for treatment groups were compared by the one-tailed Student *t*-test. In all studies, the value for statistical significance was considered to be $P \leq 0.05$.

RESULTS

UVR Induction of Slug and Snail

Our preliminary studies in vitro indicated that UVR induction of Slug mRNA reached maximal or near maximal levels at 2 h after exposure and that Snail mRNA levels were also elevated at that time point. Therefore, to investigate the dose dependence of Snail and Slug induction, we exposed SCC 12F human cells to 0, 150, 300, 600, or 1200 J/m² UVR and measured mRNA levels 2 h postexposure (Figure 1). For both Snail and Slug, mRNA induction was linearly dose-dependent up to 300 J/m² and declined at higher doses. Decreased induction at higher UVR doses was likely the result of acute UVR-induced cell damage. Although the magnitude of UVR induction differed for Slug versus Snail (3-fold vs. almost 20-fold) at maximal stimulation, Slug mRNA levels remained more than 20-fold higher than Snail levels, based on the observed 150-fold greater expression level of Slug mRNA in confluent,

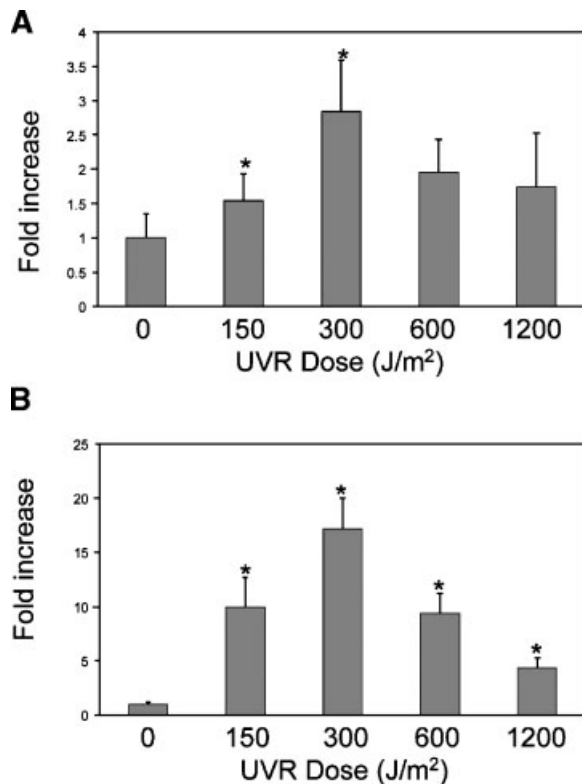


Figure 1. UVR dose dependence of Slug and Snail mRNA induction. RNA from SCC 12F cells was collected 2 h after the indicated UVR exposures, and Slug (A) and Snail (B) mRNA levels were measured as described in Materials and Methods. Values were normalized to GAPDH levels. In each experiment, the baseline level of Slug or Snail in untreated cells was defined as 1.0 and other values were expressed as fold induction. Data shown represent the mean of nine independent samples $\pm$ standard deviation examined in three separate experiments. * $P < 0.05$ compared to unexposed cells.

serum-deprived SCC 12F cells compared to Snail (6.12×10^{-3} copies of Slug per 500 ng of cDNA vs. 4.01×10^{-5} copies of Snail per 500 ng of cDNA).

To determine the time course of Slug and Snail induction by UVR, SCC 12F cells were exposed to 300 J/m² UVR, the dose of UVR shown to maximally induce both genes, then harvested at different time points postexposure (Figure 2). For both Slug and Snail, significant increases in mRNA were detected as early as 1 h after UVR exposure. For Slug, maximal induction was observed at 2 h after exposure; mRNA levels subsequently declined, but remained significantly elevated above control values for at least 24 h. A different pattern of induction was seen for Snail: average Snail mRNA levels continued to increase for at least 48 h postexposure. Increases in Slug and Snail protein levels were observed at 2–4 h after UVR exposure (Figure 3). Based on these findings, it appeared that UVR exposure-induced Slug and Snail mRNA, leading to increased protein expression. The rapidity of this response suggested the possible involvement of cytoplasmic signaling pathways in modulation of Snail and Slug levels.

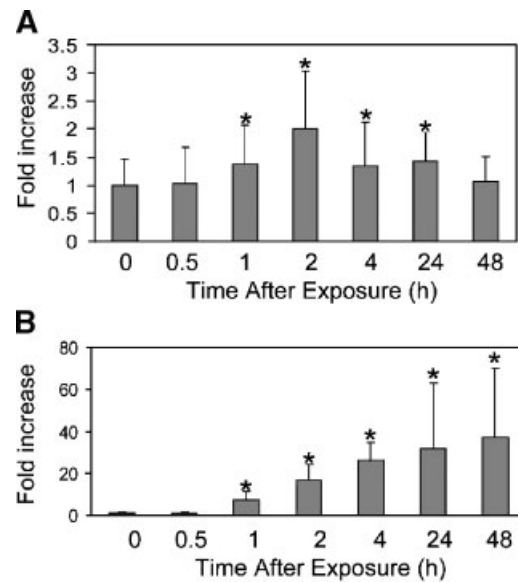


Figure 2. Time course of Slug and Snail mRNA induction. RNA from SCC 12F cells was collected at the indicated times after exposure to 300 J/m² and Slug (A) and Snail (B) mRNA levels were measured as described in Materials and Methods. For each experiment, three independent samples were included in each treatment group. Values for each sample were normalized to GAPDH levels. In each experiment, the baseline level of Slug or Snail in untreated cells was defined as 1.0 and other values were expressed as fold induction. Data shown represent the mean of nine independent samples $\pm$ standard deviation examined in three separate experiments. * $P \leq 0.05$ compared to unexposed cells.

MAPK Involvement in UVR Induction of Slug and Snail

Slug and Snail have been identified as MAPK-dependent genes [23,43–45], and UVR stimulates MAPK pathways [reviewed in Reference 30]. Because MAPK activation can vary as a function of cell type [30], we first examined the patterns of MAPK activation in SCC 12F cells in response to a dose of 300 J/m² of UVR (Figure 4). Activation of p38, JNK, and ERK by UVR was evident within 30 min postexposure, thus preceding Slug and Snail induction, and persisted for at least 2 h.

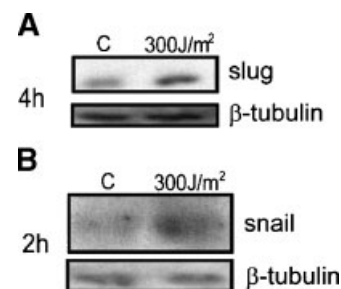


Figure 3. Detection of Slug and Snail protein following UVR exposure. Whole cell lysates were prepared from unexposed SCC12F cells (C) or cells exposed to 300 J/m² of UVR harvested at 4 (Slug) or 2 (Snail) h after exposure. Slug (A) and Snail (B) protein was detected by immunoblot analysis as described in Materials and Methods. The membranes were stripped and reprobed with antibodies against β -tubulin to ensure equal protein loading.

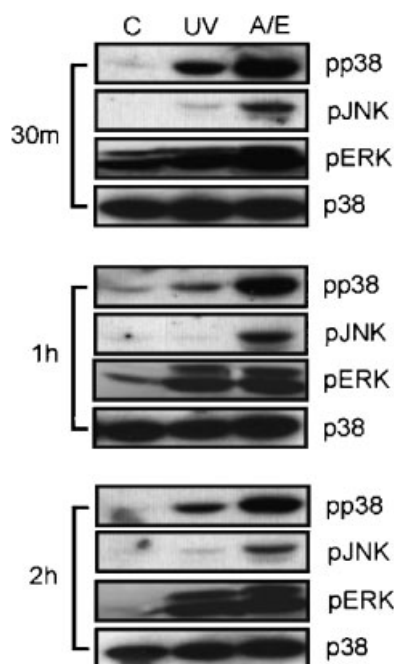


Figure 4. Activation of MAP kinase signaling cascades following UVR exposure. Whole cell lysates were prepared at the indicated time points from unexposed SCC12F cells or cells exposed to 300 J/m² or treatment with a known activator of specific MAP kinases. A = anisomycin (10 ng/mL) as an activator of the JNK and p38 MAPKs. E = EGF (20 nM) as an activator of ERK. Total protein was resolved by SDS-PAGE. The activated phosphorylated forms of p38 (pp38), JNK (pJNK), and ERK (pERK) were detected by Western blot with phospho-specific antibodies recognizing the active form of the protein. Equal protein loading was confirmed by reprobing the original membranes for total p38. Panels are representative of three or more experiments.

We then examined the role of these MAPK cascades in UVR induction of Slug and Snail. Inhibition of ERK activity with MEK1/2 inhibitor U0126 did not significantly decrease basal levels of Snail or Slug expression (Figure 5A). This inhibitor did not significantly reduce UVR induction of Slug mRNA expression compared to UVR induction in the absence of inhibitor, but significantly reduced Snail induction by UVR (~60%). Although treating unexposed cells with the p38 inhibitor SB202190 did not alter basal levels of Slug and Snail expression, pretreating cells with the inhibitor reduced UVR-stimulated Slug expression to basal levels and Snail expression by almost 70% (Figure 5B), indicating that UVR activation of p38 significantly contributed to induction of both genes. In contrast, the JNK inhibitor SP600125 significantly increased both basal and UVR-induced levels of Slug mRNA expression (Figure 5C); JNK inhibition did not significantly alter basal levels of Snail expression, but significantly enhanced UVR induction of Snail. For both Slug and Snail, JNK inhibition increased UVR induction by 5- to 10-fold. Although it did not inhibit UVR induction of Slug or Snail, SP600125 blocked anisomycin-

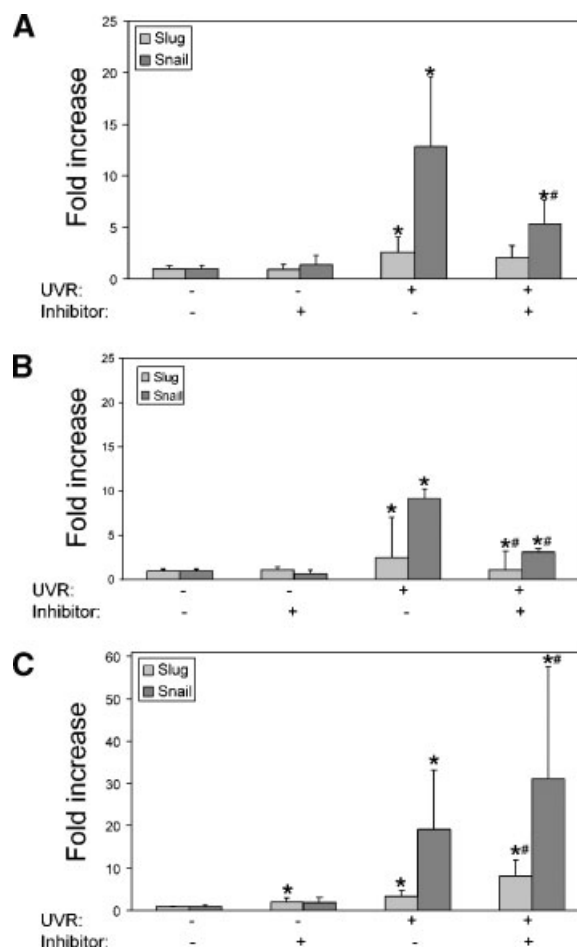


Figure 5. MAPK requirements for Slug and Snail induction by UVR. SCC 12F cells were pretreated with 10 μ M MAPK inhibitor (A = MEK1/2 inhibitor U0126 to prevent ERK activation; B = p38 inhibitor SB202190; C = JNK inhibitor SP600125) for 2 h before UVR exposure (300 J/m²). RNA was collected 2 h postexposure and mRNA levels measured for Slug (light bars) and Snail (dark bars) as described in Materials and Methods. For each experiment, three independent samples were included in each treatment group. Values for each sample were normalized to GAPDH levels. In each experiment, the baseline level of Slug or Snail in untreated cells was defined as 1.0 and other values were expressed as fold induction. Data shown represent the mean of 6–9 independent samples $\pm$ standard deviation examined in 2–3 separate experiments. * P < 0.05 compared to unexposed cells. ** P < 0.005 compared to UVR-exposed cells without inhibitor.

stimulated *c-jun* phosphorylation in SCC 12F cells (data not shown). Thus SP600125 was effective at the concentrations employed when tested with another well-established activator of JNK activity.

These findings indicate that UVR induction of Slug and Snail was mediated, at least in part, through UVR activation of p38, that Snail induction was partially dependent on ERK/MAPK pathways, and that JNK inhibition augmented UVR induction of Slug and Snail. Thus, although both p38 and JNK are stress-activated MAPK components, their roles in UVR induction of Slug and Snail are dissimilar.

Wavelength Dependence of Slug and Snail Induction In Vitro

Although the specific MAPK pathways activated by UVR and the pathways leading to this activation are somewhat wavelength-dependent, UVA, UVB, and UVC share some MAPK activation pathways [reviewed in Reference 30]. Activation of the EGF receptor appears to contribute to MAPK activation after exposure to all portions of the UVR spectrum, and activation of MAPK cascades after UVB and UVC exposure appears to be due, at least in part, to protein kinase C. An important difference among the three regions of the UVR spectrum is that shorter UVR wavelengths are much more effective at inducing DNA damage than longer wavelengths [35]; however, the role of DNA damage in initiating MAPK activation remains unclear. To determine the contribution of the small amount of UVC in the light emitted by unfiltered sunlamps, we compared Slug and Snail induction in SCC 12F cells exposed to 300 J/m² of UVR of unfiltered or Kodacel-filtered UVR. As shown in Figure 6, removing UVC wave-

lengths by Kodacel filtration reduced induction of Snail, but not Slug, significantly. However, even with removal of UVC wavelengths, Slug and Snail expression was significantly enhanced by UVR exposure.

Slug and Snail Induction by UVR In Vivo

To confirm that UVR induces Slug and Snail expression in vivo as well as in vitro, we exposed hairless mice to 1.5 MED of UVR from Kodacel-filtered sunlamps, a physiologically relevant UVR dose. In these studies, the basal level of Slug mRNA expression was more than 60-fold higher than basal Snail expression, similar to the findings obtained in vitro. Exposure to 1.5 MED of Kodacel-filtered UVR enhanced both Slug and Snail expression with delayed onset, but greater persistence, compared to in vitro findings. Slug expression was significantly increased at 24–48 h after exposure. Increasing the UVR dose to 3.0 MED slightly but not significantly increased the response detected at 24 h (Figure 7). Snail expression was significantly elevated at 2–48 h after exposure (Figure 7). Peak levels of induction for both occurred at 48 h after exposure, at which time Slug and Snail expression was increased approximately fivefold over basal levels. Thus, even at maximal induction in vivo, levels of Slug mRNA substantially exceeded Snail levels, as also seen for cultured keratinocytes. Based on these findings, it was evident that physiologically relevant UVR exposure significantly increased expression of both Slug and Snail in adult murine skin and in cultured SCC 12F cells.

Induction of Keratin 6 in Slug Knockout and Wild-Type Mice

Because the Slug transcription factor is known to regulate expression of some keratins [26,27] and

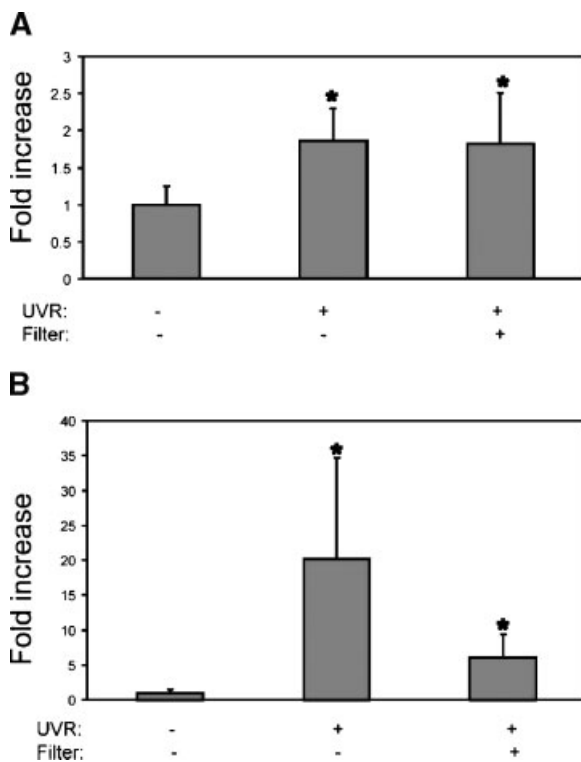


Figure 6. Wavelength dependence for Slug and Snail induction by UVR. SCC 12F cells were exposed to 300 J/m² from unfiltered or Kodacel-filtered Philips sunlamps. RNA was collected 2 h postexposure and mRNA levels measured for Slug (A) and Snail (B) as described in Materials and Methods. For each experiment, three independent samples were included in each treatment group. Values for each sample were normalized to GAPDH levels. In each experiment, the baseline level of Slug or Snail in untreated cells was defined as 1.0 and other values were expressed as fold induction. Data shown represent the mean of nine independent samples + standard deviation examined in three separate experiments. **P* < 0.05 compared to unexposed cells.

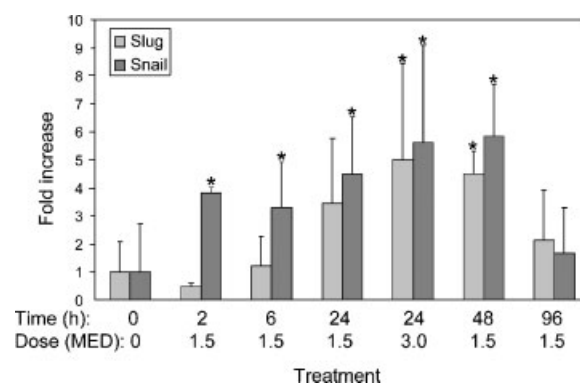


Figure 7. UVR induction of Slug and Snail in vivo. Young adult Skh-1 hairless mice were exposed to 0, 1.5, or 3.0 minimal erythral doses (MED) of UVR from Kodacel-filtered Philips sunlamps. Skin was harvested at the indicated time points, and RNA was isolated from the epidermis. Slug and Snail transcripts were measured as described in Materials and Methods. Results were normalized to the baseline levels (defined as 1.0) and expressed as fold induction. Data shown represent the mean of four individual mice per time point + standard deviation. **P* < 0.05 when compared to untreated mice by one-tailed *t* test.

because keratin 6 is both UVR-inducible and elevated in UVR-induced SCC [46,47], we examined keratin 6 expression in the UVR-exposed epidermis of Slug knockout and wild-type mice (Figure 8). Keratin 6 expression was essentially absent in the interfollicular epidermis of either wild-type or Slug knockout control mice not exposed to UVR. As expected based on previous reports [47], keratin 6 was robustly induced in all wild-type mice by 12 h after exposure to 3 MED of UVR. However, in all Slug knockout mice, there was little or no induction of keratin 6 at this time point; keratin 6 expression in Slug knockout mice did not attain the levels seen in wild-type mice until 48 h postexposure. In both wild-type and Slug knockout mice, keratin 6 expression persisted until at least 96 h after UVR exposure. These findings suggested that Slug plays a role in the physiological response to UVR by accelerating expression of keratin 6 in response to UVR exposure; however, our

results did not indicate if Slug modulated keratin 6 expression directly or indirectly or if the two were coordinately regulated.

DISCUSSION

Slug and Snail were first identified as transcription factors expressed during embryonic development and subsequently associated with tumor progression [18,20,48]. Evidence is accumulating that elevated expression of Slug or Snail in normal or tumorigenic adult tissues regulates EMT-associated responses similar to those observed in developmental processes [18,20,48]. Our present findings demonstrate that Slug and Snail mRNAs are present in untreated cultured human keratinocytes and mouse epidermis. We have shown previously that Slug expression in the epidermis is localized to islands of basal keratinocytes in the interfollicular epithelium and infundibula of hair shafts [49]. In cultured keratinocytes, Slug expression is largely restricted to migrating keratinocytes at the free edges of wounded cell sheets and expanding colonies [14]. Our present studies and those of others indicate that Slug is expressed at much higher levels than Snail in cultured murine and human keratinocytes [13,14]. Furthermore, Slug expression is transiently increased at the margins of healing wounds, where it appears to enhance desmosomal dissolution and keratinocyte motility, while changes in Snail expression levels at wound borders are minimal [14]. These findings suggest that Slug plays an important role in normal skin maintenance, renewal, and healing.

In the present studies, Slug and Snail mRNAs were rapidly induced in epidermal cells *in vitro* and *in vivo* by exposure to physiologically relevant doses of UVR. Thus, expression of Snail family transcription factors is responsive to this environmental stimulus. The kinetics of induction for Slug differed between our *in vitro* and *in vivo* studies, with UVR induction of Slug peaking at 2 h postexposure *in vitro* and at 48 h postexposure *in vivo*. The maximal extent of Slug induction, however, was similar *in vitro* and *in vivo*. On the other hand, Snail induction continued to increase until 48 h after UVR exposure both *in vitro* and *in vivo*, although the extent of Snail induction was markedly reduced *in vivo* compared to *in vitro*. These findings suggest that tissue factors released by the skin in response to acute UVR exposure may have modified Slug induction *in vivo*.

Our *in vitro* studies utilized unfiltered sunlamps that emitted 60% UVB wavelengths and 40% UVA wavelengths, with less than 3% of the radiation in the UVC range. Such lamps, both with and without Kodacel filters, have been used extensively to study UVR effects and appear to be appropriate for the biological effects of UVB [50]. However, the UVC present in unfiltered sunlamps contributes disproportionately to UVR-induced DNA damage and to

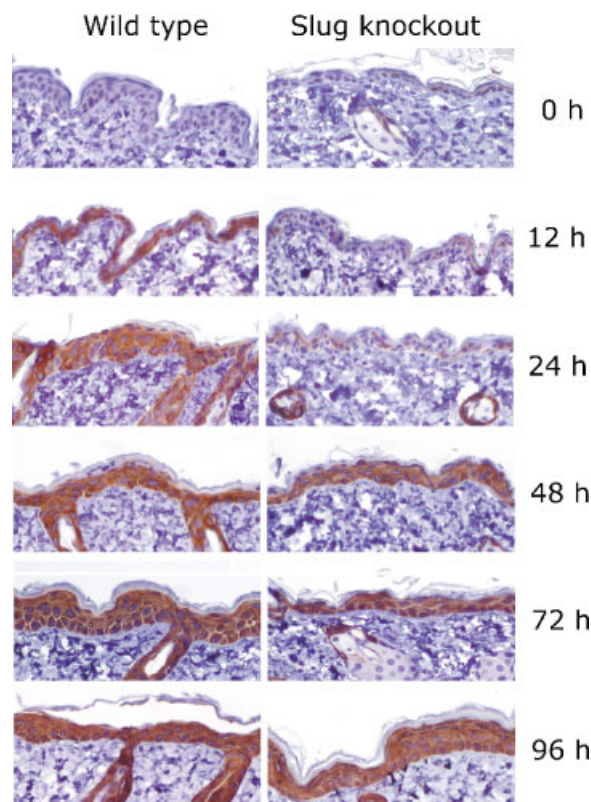


Figure 8. UVR induction of keratin 6 in Slug knockout and wild-type mice. Young adult Slug-lacZ homozygous (knockout) and their wild-type 129 littermates were exposed to 3 MED of UVB from Kodacel-filtered lamps. Twelve, twenty-four, forty-eight, seventy-two, and ninety-six hours later, skin was harvested and stained for keratin 6 as described in Materials and Methods. By 12 h post-UVR, there was prominent and diffuse keratin 6 expression in the epidermis of the wild-type mice (left panels), but there was little or no expression of keratin 6 in the epidermis of Slug knockout mice (right panels) at this time point. Keratin 6 levels in Slug knockout mice did not attain the levels observed in wild-type mice until 48 h after UVR exposure. The 0 h time point shows skin from shaved and depilated mice not exposed to UVR. Figures shown are representative of findings in three mice of each genotype.

the biological effects of this damage [34]. Interestingly, removing UVC wavelengths markedly reduced UVR induction of Snail but not Slug. Thus, although we have demonstrated that UVB and UVA wavelengths present in sunlight can enhance expression of Snail family transcription factors, the contribution of DNA damage to this process remains to be determined.

Our studies demonstrated that UVR-stimulated MAPK signaling cascades play a role in induction of Slug and Snail mRNA. We detected activation of ERK, JNK, and p38 *in vitro* in response to UVR emitted from an unfiltered sunlamp. Several studies demonstrate preferential activation of JNK and p38 by UVB in keratinocytes, and it has been reported that UVA activates all three MAPKs in these cells [reviewed in Reference 30]. Slug has previously been shown to be an ERK-regulated gene [23,43], although other pathways, such as β -catenin signaling, also exert some control over Slug expression [23]. Snail is at least partially regulated by MAPK cascades, based on the finding that Ras-mediated induction of the Snail promoter is dependent on both MAPK and phosphatidylinositol 3-kinase activities [44]. In our studies, disruption of p38 and ERK signaling impaired Slug and Snail induction by UVR; however, inhibition of JNK enhanced UVR induction of these mRNAs. Interestingly, different isoforms of JNK appear to play opposing roles in the two-stage skin carcinogenesis model [51,52], and the potential for isoform-specific regulation of Slug and Snail remains to be determined. Alternatively, based on extensive cross-talk between signaling cascades, JNK inhibition may result in the augmentation of another signaling pathway [53].

Modulation of Slug and Snail expression via MAPK pathways is consistent with present knowledge about the control of EMT in adult epithelial cells. Cell scattering has been induced in a variety of adult epithelial cells by growth factors including EGF, hepatocyte growth factor (HGF), fibroblast growth factor-1 (FGF-1), platelet-derived growth factor, and transforming growth factor- β (TGF- β), which exert their effects, at least in part, via activating Ras and downstream MAPK pathways [reviewed in References 28,29]. Several of these growth factors have been demonstrated to modulate EMT by MAPK-dependent induction of Slug or Snail. EGF, HGF, and FGF-1 induce Slug-dependent EMT in NBT-II bladder carcinoma cells [43,54], while both TGF- β and FGF-2 stimulate Snail expression and subsequent EMT in Madin–Darby canine kidney cells [44].

Rapid induction of keratin 6 transcription following UVR exposure of human keratinocytes and mouse epidermis has been reported previously [47,55]. The present studies demonstrated a potential role for Slug in this process. Expression of keratin 6 protein in the keratinocytes of wild-type mice was markedly increased at 12 h after UVR exposure;

however, similarly treated Slug knockout mice did not exhibit increased keratin 6 expression until much later (48 h). Delayed keratin 6 induction in Slug knockout mice strongly suggests a physiological role for Slug in keratinocytes in the acute UVR response. Such a role for Slug is consistent with evidence showing that Slug regulates expression of other keratins, specifically keratins 8 and 19 in human breast tumor cells [27] and that Snail regulates expression of several keratins, including keratins 7, 15, 18, 19, and 20 [19]. In these cases, however, Slug and Snail appear to repress gene expression, presumably by binding directly to consensus E-box sequences in the promoters, while in the case of keratin 6, Slug appears to be required for timely induction of gene expression. Moreover, the kinetics of Slug induction by UVR *in vivo* in our studies was not entirely consistent with the kinetics of keratin 6 induction. While Slug induction did not peak until 48 h postexposure *in vivo*, differences in keratin 6 expression in wild-type versus Slug knockout mice were evident at 12 h postexposure and disappeared by 48 h postexposure.

Our studies do not indicate if Slug regulates keratin 6 expression directly or indirectly or if Slug and keratin 6 expression is coordinately regulated via a common, as yet unidentified, pathway. There are at least 2 keratin 6 isoforms in the mouse, MK6a and MK6b, which are encoded by different genes and which appear to have evolved independently from their human keratin 6 orthologs [56]. The two murine keratin 6 genes have different regulatory regions and are differentially transcribed; they exhibit similar but not identical patterns of expression [32,56]. Examination of the 3000 bases upstream of the first exon of MK6a and MK6b as reported in ENSEMBL (<http://www.ebi.ac.uk/ensembl/index.html>) revealed consensus Snail family binding sites (CTGGAG, CTCCAG) in MK6a and MK6b promoters. In the MK6a promoter region there are five binding sites, located at –2985, –1040, –666, –653, –370 relative to the ENSEMBL-determined start of the first exon; in MK6b there are two sites, at –2954, –551 relative to the ENSEMBL-determined start of the first exon. One of the binding sites in MK6a is located more than 1.3-kb distal to the first exon, in the promoter region that confers inducibility on MK6a expression [57].

The role of keratin 6 in keratinocyte differentiation and function is complex and incompletely understood. Keratin 6 is highly expressed in proliferating epidermis and is often used as a marker of proliferation [31]. Mice that express high levels of dominant-negative MK6a constructs exhibit intraepidermal blistering and disrupted keratin filament networks, suggesting a role for keratin-6 in maintaining the mechanical strength of the epidermis [58]. Keratin 6 appears to play a role in keratinocyte migration, although its contribution to the process is not

entirely clear. MK6a/MK6b null keratinocytes migrate faster than wild-type keratinocytes in vitro; however, due to the extreme fragility of the null keratinocytes, they do not survive in the wound environment in vivo [55]. Moreover, in mice that fail to express epiplakin, a molecule functionally linked to keratin 6, keratinocyte migration is enhanced [59]. On the other hand, MK6a null mice exhibit delayed reepithelialization from the hair follicle after superficial skin wounding by tape stripping [60].

At some stage during SCC development, it seems likely that transient UVR-induced expression of Slug and Snail becomes persistent, as has been demonstrated for other transcription factors important in skin carcinogenesis, including *c-myc* and AP-1 [61]. The present studies show that Slug is transiently induced by acute UVR exposure, and studies from other laboratories indicate that Slug expression is persistently elevated in UVR-induced SCC [46,47]. Taken together, the similar pattern of transient keratin 6 expression in response to acute UVR exposure that we have demonstrated and persistent keratin 6 expression in UVR-induced SCC shown by other investigators suggest that keratin 6 expression may be linked to Slug expression.

Based on the known functions of Slug and Snail in regulation of EMT during development, increased expression of these proteins could contribute to EMT-like events in UVR-induced skin tumors. In human breast cancer, it has been established that Slug and Snail are inappropriately expressed in mammary tumors and that levels of their expression correlate with clinical disease outcome [62,63]. More salient to skin cancer are the observations that Snail and Slug are expressed in cell lines derived from chemically induced murine SCC tumors and that levels of Snail expression correlate with tumor stage [13]. It has recently been demonstrated that demethylation of the Snail promoter is associated with transition from epithelial to spindle cell morphology in chemically induced SCC in mice [16], indicating one mechanism for epigenetic regulation of Snail family expression during tumor progression.

Transiently or persistently elevated expression of Snail family transcription factors is likely to impinge on multiple aspects of skin tumor development and progression. Slug and Snail regulate cell–cell adhesion [18,20] and stimulate cell migration [14,64]. Both of these functions are central to tumor invasion and metastasis. In addition, both Slug and Snail inhibit DNA damage-induced apoptosis in a variety of cell types [65,66]. Slug protects hematopoietic stem cells against p53-independent radiation-induced cell death [65] and both Slug and Snail are anti-apoptotic in adriamycin-treated mammary carcinoma cells [66]. Based on these findings, enhanced Slug expression in the skin may prevent apoptosis in UVR-exposed keratinocytes that sustain substantial

DNA damage. This would circumvent the important tumor suppressor activity of p53 in the skin and foster skin tumor development. Our results suggest that UVR-enhanced expression of Snail family members may contribute to the development and progression of SCC. Although the precise role of these transcription factors in SCC has not been fully elucidated, they may constitute targets for preventive and therapeutic strategies aimed at decreasing SCC emergence and progression in the human population.

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