A Transgenic Mouse for Imaging Caspase-Dependent Apoptosis within the Skin

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Apoptosis is an essential process for the maintenance of normal physiology. The ability to noninvasively image apoptosis in living animals would provide unique insights into its role in normal and disease processes. Herein, a recombinant reporter consisting of β-galactosidase gene flanked by two estrogen receptor regulatory domains and intervening Asp-Glu-Val-Glu sequences was constructed to serve as a tool for in vivo assessment of apoptotic activity. The results demonstrate that when expressed in its intact form, the hybrid reporter had undetectable β-galactosidase activity. Caspase 3 activation in response to an apoptotic stimulus resulted in cleavage of the reporter, and thereby reconstitution of β-galactosidase activity. Enzymatic activation of the reporter during an apoptotic event enabled noninvasive measurement of β-galactosidase activity in living cells, which correlated with traditional measures of apoptosis in a dose- and time-dependent manner. Using a near-infrared fluorescent substrate of β-galactosidase (9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside), noninvasive in vivo imaging of apoptosis was achieved in a xenograft tumor model in response to proapoptotic therapy. Finally, a transgenic mouse model was developed expressing the ER-LACZ-ER reporter within the skin. This reporter and transgene mouse could serve as a unique tool for the study of apoptosis in living cells and animals, especially in the context of skin biology.

Journal of Investigative Dermatology (2010) 130, 1797–1806; doi:10.1038/jid.2010.55; published online 1 April 2010

INTRODUCTION

Apoptosis is a highly conserved, genetically programmed cell death process that removes unwanted or damaged cells. Apoptosis is distinguished from necrosis based on the fact that apoptosis results from activation of specific signaling pathways, which include the intrinsic and extrinsic pathways. The extrinsic pathway involves activation of cell surface death receptors (e.g., tumor necrosis factor (TNF) receptor superfamily, member 6 (FAS) and TNF-α receptors by their respective ligands), whereas the intrinsic pathway is generally mediated through mitochondrial events that lead to apoptotic peptidase-activating factor-1 and cytochrome c-mediated activation of the apoptotic machinery (Bhojani et al., 2003). In either case, activation of specific effector molecules (e.g., caspases) culminates in cell death that can be morphologically distinguished from necrosis. Apoptotic death is generally characterized by chromatin condensation, DNA fragmentation, blebbing of the cytoplasmic membrane and release of apoptotic bodies, and so on (Hengartner, 2000; Strasser et al., 2000; Bhojani et al., 2003; Nicholson and Thornberry, 2003).

The consequence of both the extrinsic and intrinsic apoptotic pathways is the activation of members of the caspase family of proteases, in particular caspase 3, which in turn can cleave a large number of intracellular substrates (Strasser et al., 2000; Botchkareva et al., 2006). A variety of stimuli, such as changes in the levels of growth factors, loss of adhesion in cells, DNA damage, or activation of proapoptotic receptors, can all initiate apoptosis (Afford and Randhawa, 2000; Botchkareva et al., 2006), and dysregulation of apoptosis can lead to either excessive elimination of cells or prolonged survival of cells. Thus, dysfunctional apoptosis can have many deleterious effects resulting in pathogenesis of various diseases such as cancer, AIDS, neurodegenerative disorders, myelodysplastic syndromes, ischemia/reperfusion injury, and autoimmune diseases (Kaufmann and Gores, 2000; Strasser et al., 2000; Shi, 2002; Bhojani et al., 2003; Nicholson and Thornberry, 2003; Loro et al., 2005).

The importance of apoptosis in self-renewal within the skin and hair in response to environmental damage, as well as...
as in diseases of keratinocytes, is now becoming apparent (Raj et al., 2006). It has been shown recently that keratinocytes possess reversible physiological defenses against spontaneous and UV light-induced apoptosis (Norris et al., 1997; Pincelli et al., 1997). Similarly, human melanocytes are protected from UV-induced apoptosis by neurotrophic proteins (Zhai et al., 1996) secreted in a paracrine manner by other skin cells (Yaar et al., 1994). In contrast, loss of programmed cell death may be involved in the development of skin cancer (Botchkareva et al., 2006). Indeed, in UV-induced skin cancer, inactivation of transformation-related protein 53 reduces the appearance of “sunburn cells,” apoptotic keratinocytes that might have incurred mutations and thus are to be eliminated (Ziegler et al., 1994). B-cell leukemia/lymphoma-2 is a major antiapoptotic protein (Kroemer, 1997) that is expressed in normal keratinocytes and melanocytes (Rodriguez-Villanueva et al., 1995). Aberrant expression of B-cell leukemia/lymphoma-2 has been involved in tumor development (Fanidi et al., 1992), and changes in B-cell leukemia/lymphoma-2 levels have been observed in melanoma (Kanter-Lewensohn et al., 1997) and nonmelanoma (Cerroni and Kerl, 1994; Morales-Ducet et al., 1995) skin cancers.

The ability to noninvasively image apoptosis would provide unique insight into the pathogenesis of the disorders referenced above. To this end, we here describe a reporter in which fluorescence imaging can be used as a surrogate for caspase activation, and therefore apoptosis. This reporter is an adaptation of a bioluminescence-based reporter described previously (Laxman et al., 2002). Owing to the lack of resolution and dependence on cofactors such as adenosine triphosphate when luciferase is used as a reporter, we have modified this reporter for imaging of apoptosis using fluorescence imaging. In this study, we describe the development of a β-galactosidase-based reporter molecule, whose activity is dependent on caspase-3 activity. In this reporter, the β-galactosidase sequence is flanked by a protease cleavage site for caspase 3 (Asp-Glu-Val-Glu) on either side of the coding sequence, which is further fused with two estrogen receptor regulatory domains (ER) on each end (Laxman et al., 2002). Expression of this reporter in cells enabled noninvasive imaging in live cells as well as in tumor xenografts and transgenic animals, in which the reporter is expressed in the skin using the keratin 5 (KRT5) promoter. These transgenic animals provide the ability to image apoptosis in a sensitive, specific, and dynamic manner, and thus can be used to obtain unique insights into the role of apoptosis in biology of the skin (e.g., in wound healing) as well as in diseases of the skin (e.g., in UV light-induced DNA damage and cellular transformation).

RESULTS
Characterization of the ER-LacZ-ER reporter
The functional basis of the ER-LACZ-ER reporter is based on the fact that β-galactosidase is active as a tetramer. We hypothesized that fusion of the ER domain at the amino- and carboxyl-termini (Figure 1a) of the protein would result in a protein, in which multimerization is stearically prevented and thus inhibits β-galactosidase activity. Proteolytic release of the ER domains in a caspase 3-dependent manner was enabled by the inclusion of the Asp-Glu-Val-Glu sequence between the ER and the β-galactosidase coding sequences, therefore enabling functional activation of β-galactosidase (Figure 1b) in the presence of active caspase 3 (during apoptosis).

To validate this approach, D54 glioma cells stably expressing the ER-LACZ-ER reporter were treated with TNF superfamily, member 10 (TNFSF10). In untreated cells (0 hour), the reporter was detected as a 190-kDa species (Figure 1c), but 1 hour after TNFSF10 treatment, the 190-kDa band dissipated with a concomitant appearance of a 150-kDa species corresponding to β-galactosidase (110 kDa) fused to a single ER (40 kDa). At later time points, the 110-kDa species of free β-galactosidase was detected. Analysis of caspase 3 activation in these samples confirmed the presence of active caspase 3 at both 1 and 2.5 hours time points.
To investigate the consequence of these caspase 3-dependent cleavage events on reporter activity, β-galactosidase activity was measured in D54 cells expressing wild-type β-galactosidase (D54/LACZ) or the ER-LACZ-ER reporter were treated in the presence of 100 ng ml⁻¹ tumor necrosis factor superfamily, member 10 (TNFSF10) or untreated (Control). At each time point, β-galactosidase activity was measured using the DDAOG (9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside) substrate and live cells. (b) Activation of β-galactosidase in D54/ER-LACZ-ER cells following TNFSF10 treatment in a dose-dependent manner. (c) Fluorescent microscopic imaging showing time-dependent activation of β-galactosidase after treatment with 200 ng ml⁻¹ of TNFSF10. The presence of the DDAOG (intact) substrate was imaged by excitation at 488 nm (pseudocolored in green), while production of the DDAO (cleaved) product in a β-galactosidase-dependent manner was imaged by excitation at 633 nm (pseudocolored in red). Bar = 20 μm. (d) A dose-dependent increase in β-galactosidase activity was determined as in panel b. Bar = 20 μm.

Figure 2b demonstrates that the activation of β-galactosidase activity in D54/ER-LACZ-ER cells was dose-dependent and that doses beyond 100 ng ml⁻¹ of TNFSF10 appear saturating. To demonstrate the use of the reporter in live cell imaging of apoptosis, D54/ER-LACZ-ER cells were treated with TNFSF10 and imaged at various times using DDAOG (9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside). DDAOG is a chromogenic substrate made by conjugating β-galactosidase and 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one (DDAO). Cleavage of DDAOG into DDAO by β-galactosidase produces a far-red fluorescent signal. The cleaved substrate DDAO emits a 50 nm red shift enabling specific detection of the cleaved...
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Figure 3. In vivo imaging of apoptosis in D54/ER-LACZ-ER xenografts. (a) D54/ER-LACZ-ER glioma cell lines were used to generate subcutaneous tumor xenografts. Fluorescence imaging was achieved using a Maestro imaging workstation (CRI, Woburn, MA) as described in the Materials and Methods section. Upon DDAOG (9H-{1,3-dichloro-9,9-dimethylacridin-2-one-7-yl}-β-D-galactopyranoside) administration, images were acquired before (T = 0 hour) and 12 hours (T = 12 hours) after treatment with saline (control) or tumor necrosis factor superfamily, member 10 (TNFSF10) treatment. Bar = 1 cm. (b) The average percentage change in fluorescence activity in saline-treated (control) and TNFSF10-treated animals was found to be statistically different (P < 0.001). Error bars represent ± SD, n = 5.

Expression of ER-LACZ-ER reporter in transgenic mice

In an effort to develop a mouse model, in which apoptosis could be monitored noninvasively and dynamically in the skin, we constructed a transgenic mouse in which ER-LACZ-ER reporter was driven by the keratinocyte-specific KRT5 promoter. The structure of the expression cassette is shown in Figure 4a. Analysis of tail genomic DNA in the founders revealed the presence of a 540 base pair sequence by PCR in transgenic animals but not control animals (Figure 4b). Immunohistochemical analysis of skin tissue from transgenic animals revealed the presence of β-galactosidase immunoreactivity in epidermal cells, which corresponded to cells that had KRT5-positive staining (Figure 4c, right). Control animals failed to show β-galactosidase-specific immunoreactivity (Figure 4c, left).

To investigate whether the reporter in the transgenic animals was conditionally activated in response to an apoptotic stimulus, shaved mice were UV-irradiated and fluorescence imaging was performed upon administration of the DDAOG substrate. No significant DDAO fluorescence was detected in mock-treated animals, but in UV-irradiated animals, a significant increase in DDAO fluorescence was observed at 24 hours compared with pretreatment (Figures 5a and b, respectively). In a cohort of five animals, a statistically significant 1.8-fold increase in DDAO fluorescence activity was detected over mock-irradiated animals (Figure 5c) (P < 0.001). Surgical removal of skin from nontransgenic as well as unirradiated (transgenic CON) and UV-irradiated (transgenic UV) transgenic animals revealed the presence of the 190-kDa ER-LACZ-ER polypeptide in the transgenic animals; it was not present in the control animals (Figure 5d). In addition, the active liberated form of the β-galactosidase (~110 kDa) was observed in the UV-irradiated animals and not in the unirradiated animals (Figure 5d). To validate the imaging studies, we conducted immunohistological studies using an antibody specific for active caspase 3 to demonstrate the presence of apoptotic activity within the UV-irradiated mouse skin samples. As shown in Figure 6, no significant staining was observed in unirradiated animals, whereas UV-treated animals had significant levels of active caspase 3 positivity within the epidermal cells.

Finally, skin sections from an untreated control mouse and a UV-irradiated mouse were stained using 5-bromo-4-chloro-
3-indolyl-β-D-galactopyranoside to identify cells that possessed both active β-galactosidase and with an antibody specific for active caspase 3. As shown in Figure 6b, untreated control cells had no significant staining for active caspase 3 and low β-galactosidase activity. These results reveal colocalization of the β-galactosidase activity with activation of caspase 3, thus directly correlating detection of the fluorescent signal with apoptosis using this molecular imaging reporter system.

DISCUSSION

Two apoptotic pathways of physiological importance have been identified in the skin. The first involves UVR in sunlight, which is the principal carcinogen, serving as initiator and promoter of most skin tumors (Ziegler et al., 1994). UV radiation elicits transformation-related protein 53-dependent apoptosis in DNA-damaged keratinocytes (sunburn cells), presumably as a “guardian of the genome” response to eradicate precancerous cells in the skin (Brash, 1996). This transformation-related protein 53-driven response, termed “cellular proofreading” (Brash, 1996), eliminates damaged cells rather than repairs damaged DNA in cells. Mice deficient in Trp53 (Trp53-null) have reduced sunburn cell formation and increased susceptibility to UV-induced skin carcinogenesis (Ziegler et al., 1994; Li et al., 1998), implicating apoptosis as a critical event in skin...
carcinogenesis. The second pathway involves activation of the cell surface death receptor FAS. Defects in this pathway have been associated with skin diseases, including eczematous dermatitis, toxic epidermal necrolysis, and graft-versus-host disease (reviewed by Raj et al. (2006)).

Orchestration of the apoptotic program is critical for the maintenance of homeostasis within the skin. The skin barrier is composed mainly of the epidermis, which is continuously renewed by the mitotic activity of stem cells in the basal layer, which provides new keratinocytes. Cornification, a programmed cell death process, involves withdrawal of keratinocytes from the cell cycle, detachment from the basement membrane, and terminal differentiation to become corneocytes in the outer layers of the epidermis, providing a critical barrier function. Imbalances in the production of keratinocytes or the formation of the cornified epithelium can disrupt the barrier function of the skin and are responsible for many skin disorders. As our understanding of the role of keratinocyte apoptosis in normal epidermal development and in various skin diseases is primarily from studies in cultured cells, validation of these concepts in animal models is needed so that apoptosis-based therapeutic interventions can be developed and validated.

In this study, we describe a mouse model in which apoptosis can be noninvasively and quantitatively evaluated macroscopically in live animals as well as microscopically in tissue sections and living cells. This will enable the translation of laboratory studies into the development of therapeutics that modulate apoptosis with the goal of treating diseases such as skin cancer, dermatitis, and graft-versus-host disease. Adapting from our previously described reporter for imaging of apoptosis using bioluminescence (Laxman et al., 2002), we present results in this study using fluorescence imaging as a readout of β-galactosidase activity that validate the specificity, sensitivity, as well as noninvasive quantitative nature of the reporter.

KRT5 is expressed in the basal layer of proliferating keratinocytes within the multilayered epithelia (Ramirez...
et al., 1994). Using the KRT5 minimal promoter fragment, we derived transgenic mice in which the above described apoptosis reporter was expressed in a tissue-specific manner. Using a KRT5-specific antibody and β-galactosidase-specific antibody, we demonstrate here that the reporter was expressed within the basal layer of the epidermis and the outer root sheath of the hair follicles (arrows) had the highest levels of active caspase 3. Bar = 40 μm. (b) Correlation of caspase 3 positivity and β-galactosidase activity. Cryosections of unirradiated (control) and UV-irradiated transgenic animals were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and an antibody specific for activated caspase 3. Colocalization of β-galactosidase activity and active caspase 3 staining was observed within the epidermal cells of treated animals (shown by arrows) but not in the control animals. Bar = 40 μm.

Figure 6. Generation of active caspase 3 within proliferating cells of the skin. (a) Immunohistological studies showing presence of active caspase 3 within the UV-treated transgenic samples (bottom) and not in mock-irradiated (control) animals (top). Proliferating populations within the skin including the basal layer of the epidermis and the outer root sheath of the hair follicles (arrows) had the highest levels of active caspase 3. Bar = 40 μm. (b) Correlation of caspase 3 positivity and β-galactosidase activity. Cryosections of unirradiated (control) and UV-irradiated transgenic animals were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and an antibody specific for activated caspase 3. Colocalization of β-galactosidase activity and active caspase 3 staining was observed within the epidermal cells of treated animals (shown by arrows) but not in the control animals. Bar = 40 μm.
morphologically the cells appeared normal, but significant β-galactosidase activity was detected in cell extracts and in live cells.

The fact that this transgenic mouse model has expression of a sensitive and quantitative reporter within the crucial cellular populations of the skin that has a key role in the biology of the skin suggests that this model will be invaluable for studies delineating the role of apoptosis in a variety of skin diseases as well as in the development and testing of therapeutic interventions for these diseases. Homeostasis between proliferation and apoptosis is crucial for optimal architecture of the skin and maintenance of epidermal barrier function. Although cornification and apoptosis is morphologically indistinguishable from the morphological features observed in traditional programmed cell death (Lippens et al., 2009), it is interesting that in the transgenic mouse expressing the reporter for caspase 3–dependent apoptosis, no significant activation of the reporter was observed in non-stimulated skin. This observation is consistent with mounting evidence that caspases 3, 6, 7, and 9 may not be involved in epidermal differentiation leading to cornification (Lippens et al., 2009). Mice lacking these caspases have essentially normal skin development. In contrast, mice lacking caspase 14, a skin-specific zymogen protease, has been implicated in skin development, as mice lacking this protein have defective corneum formation (Denecker et al., 2007). Modification of the reporter described here, so that it can be activated in a caspase 14-dependent manner, would be a valuable tool in the study of skin morphogenesis.

In conclusion, mouse models provide many opportunities to improve our understanding of the basic biology and underlying molecular mechanisms involved in the dynamic processes associated with skin injury and repair. A key component of cellular response to stress is the activation of the apoptotic machinery. To this end, we have developed a transgenic mouse model in which fluorescence activity of the skin was dependent on activation of caspase 3. Fluorescent activity after TNFSF10 treatment and UVR exposure was shown to be dose- and time-dependent and correlated directly with caspase 3 activation. Temporal evaluation of caspase 3 activity was shown to be possible by revealing the potential for using this molecular imaging reporter for a wide variety of studies; for example, involving pretreatment with UV-modifying agents. Overall, this study reports on the development and validation of a genetically engineered molecular imaging reporter construct for the detection of apoptosis in the skin of genetically engineered mice.

MATERIALS AND METHODS
Construction of hybrid molecules
All methods in this paper were approved by the Medical Ethical Committee at the University of Michigan. Briefly, two versions of the β-galactosidase molecule were cloned into pEF vector. The first consisted of wild-type β-galactosidase (pEFLacZ) and the second was chimeric fusion gene pEFERLACZER, which harbored sequences from ER (residues 281–599 of the mouse ER) at both the amino- and the carboxy-termini of the β-galactosidase with the Asp-Glu-Val-Glu sequence intervening the two domains on either side. The coding sequences (see Supplementary Figure S1 online) were cloned into the expression vector pEF, which drives transcription of the inserted coding sequence by using the elongation factor-1α promoter with the neomycin-resistance gene as bicistronic message.

Cell culture, transfection, and treatments
D54 (human glioma) cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin sulfate, 1 mM sodium pyruvate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 292 µg ml⁻¹ L-glutamine (all from Invitrogen, Carlsbad, CA) and maintained in a humidified incubator at 37 °C and 5% CO₂. The constructs described above were transfected into D54 (human glioma) cells using Fugene (Roche Diagnostic Corporation, Indianapolis, IN), and the stable clones were selected using 0.2 mg ml⁻¹ G418 (Invitrogen) and characterized for expression of the reporter by western blot analysis using total cell lysates. Specific clones were identified and selected for further study on the basis of the expression levels of the recombinant protein. Purification of TNFSF10 was described elsewhere (Chinnaiyan et al., 2000). Treatments of cells with TNFSF10 were previously described in the study by Laxman et al. (2002). In vitro activation of caspase 3 in reporter expressing D54 cells was achieved by treating cells with 200 ng ml⁻¹ TNFSF10.

In vitro activation of DDAOG
Reporter activation was measured by using the substrate DDAOG (Invitrogen), which upon cleavage produces a shifted far-red fluorescence (Tung et al., 2004). To measure cellular activation of DDAOG by β-galactosidase, D54, D54/LACZ, and D54/ER-LACZ-ER cells were seeded in 12-well plates at 75,000 per well and 175,000 cells per well for approximately 16 hours. Cells were washed three times with HBSS (Hank’s Balanced Solution) and then treated with 100 ng ml⁻¹ of TNFSF10. Following TNFSF10 treatment, DDAOG was added directly to the live cells at a final concentration of 10 μM. After incubation, the fluorescence signal was measured using a FLUOstar OPTIMA fluorescent plate reader (BMG Labtech, Cary, NC) with a 610 excitation filter and a 650 emission filter. By exciting at a wavelength greater than 600 nm, only the cleaved substrate (DDAO) was excited and measured (Tung et al., 2004).

Fluorescence imaging in vitro
Detailed cellular activation of DDAOG was monitored using a confocal microscope (Nikon D-Eclipse C1, Nikon Instruments, Melville, NY). D54, D54/LACZ, and D54/ER-LACZ-ER cells were grown on 25 mm glass coverslips in six-well plates to 80% confluency. First the cells were treated with TNFSF10 as specified and then incubated with DDAOG (10 μM) for 30 minutes at 37 degrees in HBSS. Floating cells were collected and washed three times with HBSS along with the coverslips. Confocal microscopy was performed with a Nikon D-Eclipse C1 3 Laser unit module (Spectra Physics, Santa Clara, CA, Argon 488, Green He-Ne 543, Red He-Ne 633). The argon 488 laser was used to visualize uncleaved DDAO, and the Red He-Ne 633 laser was used to visualize cleaved substrate.

Fluorescence imaging in vivo of D54/ER-LACZ-ER xenografts
Subcutaneous tumors expressing D54/ER-LACZ-ER were established by implanting 1 × 10⁷ stably transected cells subcutaneously on the
dorsal surface of the skull in athymic-nude mice (CD-1-Foxn1\sup{nu}/Foxn1\sup{nu}, Charles River Laboratory, Portage, MI). Tumors were allowed to grow to 100 mm\(^2\) in size, at which point the experiment was initiated. Before imaging, animals were anesthetized using a mixed solution of ketamine (90 mg kg\(^{-1}\)) and xylazine (10 mg kg\(^{-1}\)) given through intraperitoneal injection. The fluorescent substrate DDAOG (0.5 mg) was then injected intravenously in a vehicle solution (100 \(\mu\)l comprising of dimethylsulfoxide and phosphate-buffered saline (1:1), and fluorescence imaging was performed approximately 15–20 minutes after injection of substrate. Near-infrared reflectance imaging was performed using the Maestro system (CRI, Woburn, MA) at 0 and 12 hours time points (Tung et al., 2004) in control and treated animals. The excitation and emission filters were set at 640 and 700 nm, respectively. The acquired images were then analyzed and quantified using the software package included in the CRI system. Signal levels were normalized on the basis of the background fluorescence levels on the skin by manually placing regions of interest. After normalization, the change in signal from each animal, using the same regions of interest for pretreatment and treatment scans, was then calculated. All mouse experiments were approved by the University Committee on the Use and Care of Animals (UCUCA) of the University of Michigan.

**PCR amplification of the transgene**

The ER-LACZ-ER transgene was amplified using the Expand Long Template PCR kit (Roche Diagnostics, Indianapolis, IN). The primer pair used for amplification was ERF (5'-AAGGAAAAAGCGCGCCG CATGGGTGCTTCAGGAGAC) and ERR (3'-GCTCATCAGATCGTGTTGGGGAA). The 5.2-kb product was purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA).

**Transgenic animals**

All animals were cared for using protocols approved by the University Committee for the Use and Care of Animals. The pBK5 vector cassette containing the KRT5 promoter was kindly provided by the Dlugosz lab (University of Michigan, Ann Arbor, MI). A NotI PCR fragment containing the ER-LACZ-ER cDNA was inserted into the polylinker of the vector pBK5 that contained the 5.2-kb bovine KRT5 regulatory sequences, \(\beta\)-globin intron 2, and the 3\'polyadenylation sequences. The ER-LACZ-ER transgene was excised from the pBK5-ER-LACZ-ER cassette using AseI. The transgene was purified and used to generate transgenic animals at the Transgenic Animal Core (Van Andel Institute, Grand Rapids, MI) was performed using polyclonal rabbit anti-mouse KRT5 (Covance, Berkeley, CA) and anti-\(\beta\)-galactosidase rabbit IgG fraction (Invitrogen). The samples were analyzed using a confocal microscope (Nikon).

**Activation and measurement of apoptosis in vivo using UVB**

Patches (1 \(\times\) 1 cm) on the dorsal skin of 2- to 6-week-old mice were depilated using Nair, Princeton, NJ. After 24 hours, the exposed dorsal skin patches were irradiated at a dose of approximately 600 J m\(^{-2}\) using a UVB lamp (FS20T12-UVB, National Biological, Twinsburg, OH), ensuring that no other portion of the mouse was exposed to radiation. According to the manufacturer, this UVB bulb emitted wavelengths between 250 and 420 nm, with peak emission at 313 nm. The intensity of the UV light source was measured before each experiment using a UVX radiometer (UV, Torrance, CA). The following day, mice were anesthetized, injected with DDAOG (0.5 mg kg\(^{-1}\)) substrate 15–20 minutes before imaging (details described above). Fluorescence activity was measured using the IVIS imaging system (Caliper, Hopkinton, MA) at 0 and 12 hours time points. The excitation and emission filters were set at 610 and 650 nm, respectively. Fluorescence imaging was performed using the Living Image 3.0 software (Caliper, Hopkinton, MA) and images were acquired after a 2-minutes exposure. Signal background levels were measured by manually placing regions of interest (\(<200\) pixels) within the visible tumor margins and the adjacent skin.

**Western blot analysis**

Briefly, the mice were killed and protein extracts were prepared by homogenizing shaved skin in RIPA Buffer using a plastic homogenizer. A quantity of 40 \(\mu\)l of the crude extract was separated on Novex 4–20% Tris-Acetate SDS-PAGE gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. The membranes were blocked in 5% milk prepared in TBS-T and probed with \(\beta\)-galactosidase rabbit IgG fraction (Invitrogen) and rabbit anti-actin (Sigma-Aldrich, St Louis, MO). The secondary rabbit polyclonal antibody (Sigma, St Louis, MO) was conjugated to horseradish peroxidase and the transgene was detected by chemiluminescent horseradish peroxidase substrate.

**Immunohistochemistry analysis and 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside staining**

Immunohistochemical staining of formalin-fixed, paraffin-embedded epidermis samples (prepared by University of Michigan Comprehensive Cancer Center Tissue Core, MI) was performed using polyclonal rabbit anti-mouse KRT5 (Covance, Berkeley, CA) and anti-\(\beta\)-galactosidase rabbit IgG fraction (Molecular Probes, Eugene, CA). The reactions were visualized with goat anti-rabbit IgG labeled with Cy3 and Cy5. For active caspase 3 and 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside immunostaining, cryosections of the skin tissue were prepared using a CryoJane (Electron Microscopy, Halfield, PA), and the tissue was stained using an active caspase 3 antibody and the \(\beta\)-galactosidase staining kit (Invitrogen). The samples were analyzed using a confocal microscope (Nikon).

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**ACKNOWLEDGMENTS**

We thank Marina Grachtchouk (University of Michigan) and Bryn Eagleson (Van Andel Research Institute) for their support in this research. This work was supported by the US National Institutes of Health research grants R01CA129623 (A.R.), R21CA131859 (A.R.), U24CA083099 (B.D.R.), P50CA093990 (B.D.R.), and R01 AR045973 (A.D.).

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid
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1806 Journal of Investigative Dermatology (2010), Volume 130