Mice Lacking Smad3 Are Protected Against Cutaneous Injury Induced by Ionizing Radiation

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Transforming growth factor-β (TGF-β) plays a central role in the pathogenesis of inflammatory and fibrotic diseases, including radiation-induced fibrosis. We previously reported that mice null for Smad3, a key downstream mediator of TGF-β, show accelerated healing of cutaneous incisional wounds with reduced inflammation and accumulation of matrix. To determine if loss of Smad3 decreases radiation-induced injury, skin of Smad3+/+ [wild-type (WT)] and −/− [knockout (KO)] mice was exposed to a single dose of 30 to 50 Gy of γ-irradiation. Six weeks later, skin from KO mice showed significantly less epidermal acanthosis and dermal influx of mast cells, macrophages, and neutrophils than skin from WT littermates. Skin from irradiated KO mice exhibited less immunoreactive TGF-β and fewer myofibroblasts, suggesting that these mice will have a significantly reduced fibrotic response. Although irradiation induced no change in the immunohistochemical expression of the TGF-β type I receptor, the epidermal expression of the type II receptor was lost after irradiation whereas its dermal expression remained high. Primary keratinocytes and dermal fibroblasts prepared from WT and KO mice showed similar survival when irradiated, as did mice exposed to whole-body irradiation. These results suggest that inhibition of Smad3 might decrease tissue damage and reduce fibrosis after exposure to ionizing irradiation. (Am J Pathol 2002, 160:1057–1068)

The three mammalian isoforms of transforming growth factor-β (TGF-β1, TGF-β2, and TGF-β3) affect a number of cellular processes including growth, differentiation, apoptosis, chemotaxis, and extracellular matrix (ECM) production. One of the most extensively characterized biochemical functions of TGF-β is its role in regulating physiological and pathological inflammation and fibrosis. TGF-β has been implicated in fibrotic diseases, such as cirrhosis, glomerulonephritis, and pulmonary fibrosis, and blocking the actions of TGF-β with antibodies or antagonists abolishes the inflammation and fibrosis in animal models of many of these diseases. At the cellular level, TGF-β affects virtually all stages of the chronic inflammatory and fibrotic disease process. It is a potent chemotactic factor for monocytes, neutrophils, mast cells, and fibroblasts, active at femtomolar concentrations. After recruitment of inflammatory cells into an area of insult, TGF-β also activates neutrophils and induces macrophages to secrete cytokines, one of which is TGF-β itself. This autoduction of TGF-β is important for recruitment of additional inflammatory cells and maintaining local elevated levels of TGF-β. The TGF-β produced by macrophages can then induce matrix production by fibroblasts. The sustained expression of TGF-β is critical to the maintenance of the inflammatory and fibrotic response.

TGF-β signal through transmembrane receptors with intrinsic serine/threonine kinase activity. Binding of ligand to these heteromeric receptors induces carboxyl-terminal serine phosphorylation of a set of cytoplasmic signal-transducing proteins collectively referred to as “Smad” proteins. After activation/phosphorylation, pathway-specific Smad proteins (Smad2 and Smad3 for TGF-β signaling) heterodimerize with the common mediator Smad4 and this complex translocates to the nucleus to regulate expression of specific target genes. Smad7 (an inhibitory Smad) can disrupt signal transduction by preventing phosphorylation of Smad2 or Smad3. Mitogen-activated protein kinase pathways, induced by TGF-β or by other inputs, as well as protein kinase C activation also modulate TGF-β signaling by altering phosphorylation of Smads at sites other than the C-terminal serines phosphorylated by ligand-activated receptors.

Smad2 and Smad3, although highly homologous, have distinct modes of action. Smad3 regulates target gene activity directly by binding to DNA, whereas Smad2 activates transcription instead by binding to other DNA-binding transcription factors to modulate their activity. The different DNA-binding characteristics of Smad2 and
Smad3 result in regulation of distinct sets of target genes. The distinct activities of Smad2 and Smad3 are evidenced by the finding that targeted deletion of the Smad2 gene results in early embryonic lethality\(^{19–21}\) whereas mice null for Smad3 are viable for up to 8 months.\(^{22–24}\) Studies using fibroblasts derived from embryos null for either Smad2 or Smad3 show that TGF-\(\beta\)-mediated autoinduction and induction of c-fos are Smad3-dependent.\(^{25}\) Many genes, such as the ECM proteins collagen type I and type VII, contain AP-1 binding sites in their regulatory regions, and their induction by TGF-\(\beta\) has been shown to be Smad3-dependent.\(^{26–29}\)

Characterization of the basal phenotype of Smad3-null mice, as well as studies of incisional wound healing in these mice suggest that Smad3 has an important function in both inflammation and fibrosis \(in vivo\). Smad3-null mice die from defects in mucosal immunity suggestive of defects in neutrophil chemotaxis and consistent with the observation that the chemotactic response of Smad3-null neutrophils to TGF-\(\beta\) is impaired both \(in vivo\) and \(in vitro\).\(^{24}\) Cutaneous incisional wounds in Smad3-null mice show reduced influx of inflammatory cells, decreased accumulation of matrix, and enhanced re-epithelialization.\(^{30}\) This results, in part, from the impaired ability of Smad3-null macrophages to respond to TGF-\(\beta\) with chemotaxis and autoinduction; reduced levels of TGF-\(\beta\) in the granulation tissue then result in less ECM production. Additionally, Smad3-null keratinocytes lose their ability to be growth inhibited by TGF-\(\beta\) which contributes to the enhanced epithelialization of the wounds. Chronic inflammatory and fibrotic diseases share many common features with wound healing, except that the fibrotic process does not resolve. Because loss of Smad3 interferes with the effects of TGF-\(\beta\) on chemotaxis and autoinduction in inflammatory cells, and because induction of many ECM genes by TGF-\(\beta\) are also dependent on Smad3, we hypothesized that mice null for Smad3 will be resistant to chronic inflammation and fibrosis in which TGF-\(\beta\) has been shown to be involved.\(^{5}\)

Radiation fibrosis has features common to other fibrotic diseases, including the involvement of TGF-\(\beta\) in its pathogenesis.\(^{31}\) Ionizing radiation exposure frequently induces damage in the skin and underlying subcutaneous tissue. Early lesions are characterized by erythema, dry and moist desquamation, and ulceration resulting from the infiltration of inflammatory cells and increased vascularization. As a result of ionizing radiation, the epidermis may be hyperplastic or atrophic. Likewise, activated dermal fibroblasts are characterized by the appearance of cytoskeletal proteins similar to those involved in wound contraction, such as smooth muscle actin, and a deposition of a fibrous matrix that is abnormal in both quantity and quality. These fibrotic sequelae are an unwanted complication of radiotherapy and severely impact on a patient’s quality of life. Biopsies removed surgically from fibrotic lesions 6 months to 20 years after radiotherapy show enhanced expression of mRNA for collagen type I and type III and TGF-\(\beta\).\(^{31}\) Similarly, in an experimental model of cutaneous radiation in the pig, TGF-\(\beta\) mRNA was increased at 1 to 12 months after irradiation\(^{32}\) and immunoreactive TGF-\(\beta\) was localized to myofibroblasts, endothelial cells, and the collagen matrix. Irradiation of mouse skin also shows increased expression of TGF-\(\beta\) mRNA from 6 hours to 9 months after irradiation.\(^{33}\) Irradiation of other tissues such as lung,\(^{34}\) intestine,\(^{35}\) bladder,\(^{36}\) and liver\(^{37}\) is also associated with increased TGF-\(\beta\) expression.

Because elevated levels of TGF-\(\beta\) are associated with radiation-induced inflammation and fibrosis, and because Smad3-null mice show reduced inflammation, TGF-\(\beta\), and matrix accumulation after wounding, we hypothesized that loss of Smad3 will decrease inflammation and subsequent fibrosis induced by irradiation. To test this hypothesis, we exposed the skin of Smad3\(+/-\) (WT), \(+/-\) (Het), and \(-/-\) (KO) mice to high doses of \(\lambda\)-irradiation. Here we report that 6 weeks after irradiation skin from KO mice shows significantly less epidermal acanthosis, dermal inflammation, and immunoreactive TGF-\(\beta\) than does skin from WT mice.

### Materials and Methods

#### Generation of Mice

Smad3\(^{ex8/ex8}\) mice were generated by targeted disruption of the Smad3 gene by homologous recombination. Targeted embryonic stem-cell clones were microinjected into C57BL/6 blastocysts to obtain germline transmission. Mice heterozygous for the targeted disruption were intercrossed to produce homozygous offspring.\(^{24}\)

#### Whole-Body Irradiation

The protocol used in this study was institutionally approved and in accordance with guidelines of the Institute of Laboratory Animal Resources, National Research Council. Animals were exposed to whole-body irradiation using a \(^{137}\)Cesium Gamma Cell 40 (Nordion Int. Inc., Kanata, Ontario) irradiator that had been calibrated with thermoluminescent dosimetry chips (Bicron, Inc., Solon, OH) planted in phantom Plexiglas mice. Animals were irradiated between 7 to 9 weeks after birth. Smad3 WT, Het, and KO mice were placed in circular Lucite containers with holes for ventilation (up to five animals). The container was positioned within the irradiator and exposed for varying times to deliver the desired dose (single radiation doses ranging from 6 to 10 Gy at a dose rate of 0.98 Gy/minute). After irradiation, mice were removed from the container and returned to the cages in which they were housed (five animals/cage) in a climate-controlled environment with free access to food and water. If at any time an animal was unable to acquire food or water because of illness, the animal was euthanized per protocol. Mice were observed daily for survival.

#### Local Irradiation

Experiments also involved local irradiation to the leg or flank. For these studies animals (without anesthetics) were placed in customized Lucite jigs that allow for immobilization and selective irradiation of the leg or skin of
the flank. Special clamps held the skin without decreasing blood flow to the treatment area as determined previously by laser Doppler studies. Single radiation doses ranging from 30 to 60 Gy were delivered by a Therapax DXT300 X-ray irradiator (Pantak, Inc., East Haven, CT) using 2.0-mm Al filtration (300 kvp) at a dose rate of 1.9 Gy/minute. Special care was taken to avoid irradiation of other body parts by using lead shields specifically designed as a part of the jigs. In addition to WT, Het, and KO mice, four mice each of strains C57BL/6nCr. Tac:N:NIHS-BCFB/R, 129S6/SvEv-ATM<tm1awb>+, and WBB6F1/J KIT/KIT<sup>W64A</sup> (mast cell deficient) also received hind leg irradiation. After irradiation, the animals were placed in cages as indicated above and observed daily for 5 to 6 weeks at which time animals were euthanized and skin was removed from the hind legs or flank and transferred to 10% neutral buffered formalin for 24 hours. After three washes in 70% ethanol, tissues were processed for paraffin embedding.

Quantitative Histopathology and Morphometry

Formalin-fixed, paraffin-embedded tissues were cut at 5 μm and sections were stained with hematoxylin and eosin (H&E), Geimsa (to identify neutrophils), or low pH toluidine blue (to identify mast cells). The numbers of neutrophils and mast cells were counted in 5,400 magnification fields/skin section.

Macrophages, blood vessels, and myofibroblasts were identified by staining with rat anti-mouse Mac-3 (BD PharMingen, San Diego, CA), rat anti-mouse CD31 (PECAM-1) (BD PharMingen), and mouse anti-smooth muscle actin Ab-1 (NeoMarkers Inc., Fremont, CA), respectively. Staining was performed using the OptiMax Plus 2.0 Automated Cell Staining System with research software (BioGenex, San Ramon, CA). For anti-Mac-3 staining, tissue sections were deparaffinized, treated with 1% H<sub>2</sub>O<sub>2</sub> and nonspecific protein binding was blocked for 1 hour with a solution containing 1% bovine serum albumin and 5% rabbit serum. Sections were incubated for 2 hours at 37°C with anti-Mac-3 (2.5 μg/ml) in Tris-buffered saline, pH 7.4/1% bovine serum albumin. Antigen-antibody complexes were detected using the Vectastain Elite rabbit ABC peroxidase kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. After 30 minutes of incubation with biotinylated secondary antibody followed by a 30-minute incubation with ABC reagent, a 5-minute reaction with diaminobenidine/H<sub>2</sub>O<sub>2</sub> was used to detect the bound peroxidase. Staining with anti-CD31 was similar except that sections were also pretreated for 30 minutes with 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) and the primary antibody incubation was performed overnight at 4°C at 6.5 μg/ml. Staining for anti-smooth muscle actin was performed with the Vector M.O.M. kit (Vector Laboratories) according to the manufacturer’s instructions using a 2-hour incubation with 0.5 μg/ml of smooth muscle actin IgG. For all staining experiments isotype-matched normal IgG at the same concentration as the primary antibody was used as a negative control. The numbers of macrophages, blood vessels, and myofibroblasts were counted in 5,400 magnification fields/skin section.

Quantitation of epidermal thickness was done by capturing images of H&E-stained sections (×400 magnification) from a Zeiss Axioplan microscope using an MTI 3 charge-coupled device camera. On each section three areas of interfollicular acanthosis were identified by eye and the thickness of the epidermis from the outer edge to the epidermal-dermal interface was measured using ImagePro 2.0 software.

Quantitative results are expressed as mean ± SE mean. Significant intergroup differences were determined by applying the two-sample assuming unequal variance t-test.

TGF-β Ligand and Receptor Levels

Immunoreactive TGF-β1, TGF-β2, and TGF-β3, as well as the type I (RI) and type II (RII) receptors were localized to tissue sections using a protocol similar to that described above for staining with the Mac-3 antibody with the following modifications. Sections were pretreated for 30 minutes with 1 mg/ml of bovine testicular hyaluronidase (Sigma) for TGF-β ligand staining, the primary antibody incubation was performed for 2 hours at room temperature, and a Vectastain Elite rabbit ABC peroxidase kit was used. The primary antibodies used to detect TGF-β1 were IgG fractions of LC 1-30-1 (6 μg/ml) and CC 1-30-1 (0.2 μg/ml), which recognize intracellular and extracellular TGF-β1, respectively. Affinity-purified anti-TGF-β2 (catalog no. sc-90; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-TGF-β3<sup>41</sup> were used at 0.8 μg/ml and 4 μg/ml, respectively. RI and RII were detected with antibodies from Santa Cruz Biotechnology (catalog no. sc-398 and sc-220) that were used at 4 and 0.8 μg/ml, respectively.

Preparation of Keratinocytes and Fibroblasts

Epidermal keratinocytes were isolated from polymerase chain reaction-genotyped Smad3 WT and KO newborn mice by standard methods<sup>42</sup> and were cultured in Eagle’s minimal essential medium/8% Chelex-treated fetal bovine serum/0.05 mmol/L CaCl<sub>2</sub> with antibiotics. Fibroblasts were isolated by collagenase (Life Technologies, Inc., Grand Island, NY) digestion of the pooled dermal layers from the newborn WT and KO pups according to Lichti and colleagues<sup>43</sup> and were cultured in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum/1% Pen-Strep.

Cell Survival Assay

Epidermal keratinocytes were plated and cultured for 2 days before being exposed to 0, 5, or 10 Gy of γ-irradiation from a 60Co source. Cells were cultured for an additional 48 hours at which time cells were trypsinized and counted using a hemocytometer after trypan blue staining. The number of surviving unirradiated cells was set as 100% and the surviving irradiated cells were referenced to the unirradiated cells.
Dermal fibroblasts (passage 2) isolated from WT and KO mice were removed from the tissue culture flask with 0.05% trypsin-ethylenediaminetetraacetic acid (Life Technologies, Inc.) and divided into six equal aliquots. One aliquot was exposed to each of 0.5, 2, 5, and 10 Gy of γ-irradiation, whereas two aliquots were left unirradiated. Equal numbers of cells from each aliquot were plated into 35-mm dishes and allowed to grow for 5 days and then were rinsed, trypsinized, and the number of surviving cells in each dish determined with a Coulter counter. The number of surviving unirradiated cells was set as 100% and the percentage of surviving irradiated cells was compared to this.

### Results

**Loss of Smad3 Decreases Irradiation-Induced Skin Damage**

Based on our previous studies demonstrating that Smad3-null mice show accelerated healing of incisional wounds, we hypothesized that loss of Smad3 might improve healing or protect against tissue damage resulting from other types of insults. To test this hypothesis, the hind legs of a group of WT, Het, and KO mice were exposed to 50 Gy of γ-irradiation, whereas two aliquots were left unirradiated. The epidermis of KO mice showed more severe damage than that in WT animals (Figure 1B) in which a higher percentage of animals show more severe damage (ulceration and severe acanthosis) (Table 1). Samples from Het mice have an intermediate histological appearance. The epidermal thickness of the restricted set of samples that were not ulcerated showed no significant differences among the genotypes because of the severity of the damage (data not shown).

Irradiated skin from KO animals also shows less severe inflammation in the dermis than does skin from WT animals (Table 1). The severity of inflammation was graded based on the numbers of inflammatory cells in the dermis and on the density of the dermal matrix. In quantitative studies there were no significant differences in numbers of dermal neutrophils or macrophages between WT and KO mice, however there were significant differences in the numbers of mast cells (112 ± 11 for WT versus 60 ± 3 for KO, *P* < 0.001 per five high-power fields).

Because the Smad3-null mice are carried on a mixed background of three strains, we irradiated legs of mice of each individual strain (C57BL/6Ncr, Tac:NiHS-BCFBR, and 129S6/SvEv-ATM<tm1awb>) to test whether any of these strains might show increased resistance to irradiation. Irradiated skin from all three strains showed an acanthotic epidermis and severe dermal inflammation (data not shown). This assured that the protective effect seen in the KO animals is not the result of increased representation of a radio-resistant strain.

To obtain a greater area of irradiated skin for analysis, in subsequent experiments the site of irradiation was changed to the flank skin, again with care to shield the rest of the mouse from systemic irradiation. Additionally, the dose of γ-irradiation was decreased to 30 Gy to reduce ulceration of the WT skin and to facilitate quantitative analysis of the protective effects associated with loss of the Smad3 gene. Thirty Gy caused less damage to the skin resulting in only acanthosis of the WT epidermis at 6 weeks with none of the ulceration that had been observed at the 50-Gy dose. The 30-Gy dose also caused moist desquamation that peaked at ~30 days, but had resolved before the time of analysis. Again, the epidermis of skin from irradiated WT mice (Figure 1, A and G) is more hyperplastic than that from KO mice and is characterized by hyperkeratosis, parakeratosis, and keratin whorls that are absent from irradiated KO skin.

### Table 1. Qualitative Analysis of Histological Appearance of Irradiated Leg Skin Samples (50 Gy) from WT, Het, and KO Mice

<table>
<thead>
<tr>
<th>Number of animals/total (%)*</th>
<th>WT</th>
<th>Het</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulceration</td>
<td>10/20 (50)</td>
<td>10/26 (38)</td>
<td>4/22 (18)</td>
</tr>
<tr>
<td>Severe acanthosis</td>
<td>5/20 (25)</td>
<td>8/26 (31)</td>
<td>8/22 (36)</td>
</tr>
<tr>
<td>Moderate acanthosis</td>
<td>5/20 (25)</td>
<td>7/26 (27)</td>
<td>7/22 (31)</td>
</tr>
<tr>
<td>Normal</td>
<td>0/20 (0)</td>
<td>1/26 (4)</td>
<td>3/22 (14)</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>11/20 (55)</td>
<td>12/26 (46)</td>
<td>5/22 (22)</td>
</tr>
<tr>
<td>Moderate</td>
<td>9/20 (45)</td>
<td>12/26 (46)</td>
<td>13/22 (59)</td>
</tr>
<tr>
<td>Mild</td>
<td>0/20 (0)</td>
<td>2/26 (8)</td>
<td>4/22 (18)</td>
</tr>
</tbody>
</table>

*Evaluation based on area of most severe response in section.

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Figure 1. H&E staining of mouse skin after exposure to γ-irradiation. As compared to unirradiated skin (A), skin from the leg of KO (B) and WT (C) mice 5 weeks after 50 Gy of irradiation shows evidence of tissue damage that is more severe in WT (C) mice (epidermal ulceration with severe dermal inflammation and increased dermal density) than in KO (B) mice (moderate acanthosis and dermal inflammation). Keratin whorls are marked by asterisk in B. Images are representative of an experiment irradiating hind legs of 10 WT, 13 Het, and 11 KO mice. Leg skin from WBB6F1/J KIT/KITW-v mice (D) 5 weeks after 50 Gy of irradiation is totally ulcerated with an escar (arrow) and severe dermal inflammation. Six weeks after 50 Gy of irradiation there is significantly more epidermal acanthosis with parakeratosis (arrowhead), keratin whorls (asterisk), and dermal inflammation in flank skin from WT mice (E and G) as compared to KO mice (F and H). Images are representative of an experiment irradiating flanks of seven WT, six Het, and nine KO mice. Original magnifications ×100.
motaxis. However, the basal levels of macrophages in a Smad3-mediated mechanism than is macrophage che-

Figure 2. The average interfollicular epidermal thickness of flank skin 6 weeks after 30 Gy of irradiation is greater in WT and Het mice than in KO mice or in nonirradiated skin (WT-non and KO-non). Results are the average of three measurements from 12 WT, 7 Het, and 14 KO (irradiated) and 6 WT and 5 KO (nonirradiated) sections. P < 1 × 10−3 for WT versus WT-non, KO versus KO-non, WT versus KO, WT versus Het, KO versus Het.

(Figure 1, F and H). Quantitation of the interfollicular epidermal thickness is shown in Figure 2. Skin from non-

irradiated WT and KO mice exhibits similar epidermal thickness before irradiation, whereas the fold-increase after exposure to 30 Gy of irradiation is 8.0-, 4.8-, and 2.4-fold for WT, Het, and KO skin, respectively.

Similar to that reported previously in wound-healing studies,30 the dermis of the irradiated skin from WT mice has a greater density of inflammatory cells than does the dermis of KO mice. Table 2 shows that there are approximately twofold more mast cells, macrophages, and neutrophils present in the dermis of irradiated skin from WT mice as compared to KO mice. Additionally, there are 70% more blood vessels and myofibroblasts in the irradiated skin from WT animals than from KO animals. Interestingly, the numbers of mast cells in the dermis of KO mice remain unchanged after irradiation (Table 3), whereas irradiation induces an increase in numbers of mast cells in the dermis of WT mice. There is a greater fold-increase of neutrophils after irradiation in KO mice as compared to WT animals, whereas the fold-increase of macrophages is similar in both genotypes (Table 3). These data suggest that in dermal inflammation induced by irradiation, the chemotaxis of mast cells and neutrophils into the irradiated area may be more dependent on a Smad3-mediated mechanism than is macrophage chemotaxis. However, the basal levels of macrophages in unirradiated KO skin are considerably reduced compared to those in WT skin. Additionally, the greater increase in myofibroblasts in WT mice after irradiation compared to KO mice, suggests that Smad3 may also play a role in the recruitment of fibroblasts in response to TGF-

β.11 Conversion of quiescent fibroblasts to activated myofibroblasts is also thought to be a TGF-β-dependent process,44–46 but the role of Smad3 is not known.

To determine whether the reduced numbers of mast cells present in the KO skin after irradiation had a causative role in the decreased tissue damage, we irradiated the legs of mast cell-deficient mice (WBB6F1/J KIT/KITW-v). Five weeks after irradiation, skin from the legs of these animals showed ulceration of the epidermis and scar formation along with severe dermal inflammation (Figure 1D), suggesting that the absence of mast cells did not protect against radiation-induced injury despite the fact that mediators released from mast cells have been proposed to promote epidermal hyperplasia.47

Table 2. Quantitative Analysis of Flank Skin from Irradiated (30 Gy) and Nonirradiated WT, Het, and KO Mice

<table>
<thead>
<tr>
<th></th>
<th>Nonirradiated</th>
<th>Irradiated</th>
<th>Nonirradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Het</td>
<td>KO</td>
</tr>
<tr>
<td>Mast cells</td>
<td>57 ± 4 (6)*</td>
<td>112 ± 11 (16)</td>
<td>89 ± 20 (9)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>11 ± 1 (6)*</td>
<td>85 ± 11 (12)</td>
<td>60 ± 9 (11)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>7 ± 1 (6)*</td>
<td>42 ± 6 (12)</td>
<td>26 ± 4 (7)</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>55 ± 3 (6)</td>
<td>56 ± 7 (16)</td>
<td>39 ± 2 (10)</td>
</tr>
<tr>
<td>Myofibroblasts</td>
<td>10 ± 1 (6)*</td>
<td>80 ± 6 (17)</td>
<td>64 ± 6 (9)</td>
</tr>
</tbody>
</table>

*P < 0.005 versus irradiated WT.
†P < 0.02 versus irradiated WT.
‡P < 0.05 versus irradiated KO.
§P < 0.005 versus irradiated KO.

TGF-β Ligand and Receptor Expression in Irradiated Skin

Because TGF-β1 levels have been observed to increase in tissues after injury or in areas showing inflammation,4,5 and because cells that are null for Smad3 are unable to autoinduce TGF-β1,25 we compared the intensity of the immunohistochemical staining of the three TGF-β isoforms in irradiated flank skin from WT and KO mice. A summary of the changes in immunoreactivity of the different TGF-β isoforms in WT and KO skin after irradiation is shown in Table 4. Two different antibodies were used for localization of TGF-β1, LC 1-30-1 and CC 1-30-1, which recognize intracellular TGF-β1 and TGF-β1 associated with ECM, respectively.40 There is significantly more immunoreactive TGF-β1 associated with the ECM in the dermis of irradiated WT skin as compared to irradiated skin from KO mice (Figure 3, A and B). Figure 3B demonstrates that even in an area exhibiting epidermal hyperplasia in a KO mouse, there is very little immunoreactive TGF-β1 in the underlying dermis (Figure 3B, arrow). Additionally, keratinocytes in the irradiated epidermis and inflammatory cells in the dermis show more staining for intracellular TGF-β1 in WT as compared to KO mice (Figure 3, C and D). Regardless of which
TGF-β1 antibody is used, there is more staining in irradiated skin than in nonirradiated skin, but the difference in staining intensity is consistently greater with the WT mice than the KO mice (data not shown).

Dermal cells also stain more strongly for both TGF-β2 and TGF-β3 in the WT as compared to KO mice (shown for TGF-β3 in Figure 3, E and F). There are interesting differences in the expression patterns of TGF-β2 in the keratinocytes of irradiated WT and KO skin. In WT irradiated skin, immunoreactive TGF-β2 is confined to the outer layers of the epidermis (stratum granulosum), whereas in irradiated KO skin, staining is also apparent in the stratum spinosum (Figure 3, G and H). The staining pattern in the irradiated KO is similar to that seen in nonirradiated WT and KO skin where there is relatively uniform staining of the epidermis with anti-TGF-β2 (data not shown). In contrast to the increased epidermal staining for TGF-β1 after irradiation, levels of immunoreactive TGF-β3 in the epidermis decrease in both irradiated WT and KO mice as compared to nonirradiated skin (data not shown).

We also compared the immunohistochemical staining of TGF-β type I (TβRI) and II (TβRII) receptors in skin before and after irradiation to determine which cells were capable of responding to TGF-β. In both WT and KO nonirradiated skin there is light uniform staining for TβRI in the epidermis with darker staining of dermal cells and hair follicles. This pattern does not change after irradiation of either genotype (data not shown). There is, however, a dramatic change in TβRII expression after irradiation (Figure 4). Although normal skin of both WT and KO mice show strong expression of TβRII in the epidermis and hair follicles, and many dermal cells (Figure 4, A and B), most staining in the epidermis and hair follicles is lost 6 weeks after irradiation, even though staining remains high in the dermis (Figure 4, C and D). This pattern of loss of TβRII is observed in both genotypes.

### Table 3. Fold-Change in Cell Numbers in Irradiated versus Nonirradiated Skin

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast cells</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>7.7</td>
<td>6.5</td>
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<tr>
<td>Neutrophils</td>
<td>6.0</td>
<td>1.9</td>
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<tr>
<td>Blood vessels</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Myofibroblasts</td>
<td>8.0</td>
<td>4.5</td>
</tr>
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</table>

Smad3 Has No Effect on Survival after Irradiation

To examine whether the observed differences in the histology of the skin from WT and KO mice after irradiation might be based, in part, on a genotypic difference in survival of cells after exposure to irradiation, we isolated primary keratinocytes and dermal fibroblasts from 1- to 3-day-old WT and KO mice and exposed the cells to various doses of γ-irradiation. There is no significant difference in survival between WT and KO keratinocytes (Figure 5A) and fibroblasts (Figure 5B) when evaluated 2 and 5 days after irradiation, respectively. We also examined the survival of WT and KO mice after whole-body irradiation. There is no significant difference in survival of WT, Het, and KO mice 30 days after whole-body irradiation at doses of 6, 7, 8, or 10 Gy. A dose of 8 Gy is lethal for all genotypes (data not shown), suggesting that the loss of Smad3 does not afford protection from bone marrow toxicity.

### Discussion

In this study we show that disruption of the Smad3 gene affords significant protection of mice from the acanthosis, ulceration, hyperkeratosis, and dermal inflammation resulting from exposure of the skin to high doses of ionizing irradiation. This protection is associated with decreased influx of inflammatory cells and decreased expression of TGF-β isoforms in the irradiated KO skin as compared to the irradiated WT skin at 6 weeks after irradiation. Given the extensive literature linking increased expression of TGF-β to many forms of fibrosis, including radiation fibrosis, this observed decrease in TGF-β expression in KO skin after irradiation is suggestive of these mice would be less susceptible to fibrotic sequelae. The time point used for these studies was not sufficiently protracted to examine directly the ongoing chronic effects of radiation-induced fibrosis because Smad3 KO animals have a shortened life span.

Exposure to ionizing radiation has been reported to increase production of both total and active TGF-β within several hours after exposure and persisting for months. Similar to the mechanisms we have proposed to contribute to the reduced expression of TGF-β1 in granulation tissue of KO mice after wounding, we suggest that the impaired autoinduction of TGF-β1 in Smad3-null macrophages and fibroblasts, most likely accounts for a significant portion of the difference in expression of TGF-β1 after irradiation. Although Smad2-null mice are not viable, comparison of wound healing in Smad2 and Smad3 heterozygous mice showed that the reduced influx of inflammatory cells and reduced accumulation of matrix proteins were Smad3-dependent and not Smad2-dependent. Studies using mouse embryo fibroblasts null for either Smad2 or Smad3 also showed that autoinduction of TGF-β1 selectively requires Smad3 and not Smad2. Together, these results suggest that selective ablation of Smad3 may both eliminate or reduce amplification and sustained expression of TGF-β and chemotactic responses to TGF-β, thus having particular importance for fibrotic processes.

### Table 4. Qualitative Changes in Immunohistochemical Staining Intensity of TGF-β Isoforms of Flank Skin 6 Weeks After 30 Gy

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Localization</th>
<th>WT Rad/Non</th>
<th>KO Rad/Non</th>
<th>WT Rad/ KO Rad</th>
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</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>Epidermis</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>TGF-β1</td>
<td>Dermal cells</td>
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<tr>
<td>TGF-β1</td>
<td>ECM</td>
<td>↑</td>
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<tr>
<td>TGF-β2</td>
<td>Epidermis</td>
<td>↑</td>
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<td>TGF-β2</td>
<td>Dermal cells</td>
<td>↑</td>
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<tr>
<td>TGF-β3</td>
<td>Epidermis</td>
<td>↑</td>
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<tr>
<td>TGF-β3</td>
<td>Dermal cells</td>
<td>↑</td>
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Abbreviations: Rad, irradiated; Non, nonirradiated; ECM, extracellular matrix.
Two hypotheses can be put forth to explain the phe-notypic differences observed in the WT and KO mice after irradiation. On the one hand, loss of Smad3 may alter early signaling events induced by irradiation resulting in different outcomes at later time points. Alternatively, the initial signaling events after irradiation may be independent of the Smad3 status, but the inability of the Smad3-null mice to chronically express and amplify TGF-β1 in response to these signals may subsequently lead to a resolution of the tissue reaction in KO mice and ultimately to reduced fibrosis. We are currently analyzing samples at a variety of time points after irradiation to help differentiate between these two possibilities, but the data on hand, support the second hypothesis.

Ionizing radiation activates a complex array of cellular signal transduction pathways including AP-1, nuclear factor-κB, mitogen-activated protein kinases, phosphati-dyl inositol-3-phosphate kinase (PI-3 kinase), as well as DNA-dependent protein kinase (DNA-PK), poly(ADP-ribose) polymerase (PARP), and ATM (ataxia-telangiectasia-mutated) that act upstream of p53.49–52 To address the first hypothesis, we attempted to determine whether any of these signaling pathways is altered in Smad3 WT and KO skin at early times after irradiation. Our initial in vitro analyses of irradiated WT and KO primary cultures of keratinocytes and dermal fibroblasts show no difference in activation of AP-1, nuclear factor-κB, mitogen-activated protein kinases, or p53 phosphorylation (K. Flanders and M. Fujii, unpublished observations). However, study of these cell types in isolation may not appropriately model early signaling events as occur in vivo. Additionally, the activation of a multiplicity of transcription factors and signaling pathways by radiation leads to the induction of a variety of growth factors and cytokines including TGF-β, tumor necrosis factor-α, interleukin-1, fibroblast growth factor, and platelet-derived growth factor that could then secondarily affect signaling.53 We have shown that Smad3 KO skin produces less TGF-β.

Figure 3. Immunohistochemical localization of TGF-β1 in irradiated flank skin of Smad3 WT and KO mice 6 weeks after 30-Gy exposure. Staining with anti-CC 1-30-1 demonstrates extensive localization of ECM-associated TGF-β1 in the dermis of WT skin (A), whereas the only staining in the KO skin is localized to the epidermal-dermal junction (arrow). With anti-LC 1-30-1 there is intense staining for TGF-β1 in the hyperplastic keratinocytes of the WT mice (C), whereas the epidermis of KO mice shows light, uniform staining with this antibody (D). There is also stronger staining in pockets of cells in the dermis of the WT skin (arrow in C) compared to the KO skin (D). More intense immunoreactivity is observed in dermal cells of WT mice (E) as compared to KO mice (F) when stained with anti-TGF-β3. Strong staining with anti-TGF-β2 is seen in the stratum granulosum (arrow in G) of WT skin, whereas staining in the skin of the KO is more uniform and is also present in the stratum spinosum (arrow in H). There is also greater staining with anti-TGF-β2 in the dermis of the WT mice (G) as compared to the KO mice (H). Peroxidase with Carazzi hematoxylin counterstain. Original magnifications, ×100 (A–D, G, H), ×400 (E, F).

Figure 4. Immunohistochemical localization of TGF-βRII in irradiated flank skin of Smad3 WT and KO mice 6 weeks after 30-Gy exposure. Intense immunoreactivity is observed in epidermis, hair follicles, and dermal cells of nonirradiated WT (A) and KO (B) skin. After irradiation staining decreases dramatically in the epidermis, but remains strong in dermal cells in both WT (C) and KO (D) skin. Peroxidase with Carazzi hematoxylin counterstain. Original magnifications, ×200.
after irradiation and that KO cells respond differently to TGF-β than do WT cells. However, the cross-talk between the Smad and mitogen-activated protein kinase signaling pathways also raises the possibility that Smad3 WT and KO cells may differ in their production of or response to cytokines other than TGF-β. To address this, we are currently characterizing the cytokine profile of WT and KO skin after irradiation.

Immunohistochemical staining shows a dramatic decrease in the expression of TβRII in the epidermis in both WT and KO mice after irradiation, while its expression remains high in dermal cells. These effects are selective for the ligand-binding TβRII as no changes after irradiation were observed for TβRI. Recently, it has been reported that ultraviolet radiation has similar effects on mink lung epithelial cells in vitro, resulting in down-regulation of TβRII without altering levels of TβRI protein. Selective loss of TβRII expression has also been reported in a number of pathological conditions including carcinomas and in atherosclerotic lesions. In tumor cells, where effects of receptor loss have been studied extensively, it is clear that except in the rare cases of biallelic mutation that result in complete loss of TGF-β signaling, epigenetic mechanisms contributing to receptor loss likely alter, rather than completely ablate, TGF-β signaling. Typically, such receptor down-regulation results in loss of the growth inhibitory effects of TGF-β while still allowing for signaling to other target genes that may have a lower signaling threshold. In the case of loss of TβRII in epidermal keratinocytes after irradiation, it could be argued that this would ablate the potent growth inhibitory effects of TGF-β normally seen in these cells and in that manner contribute to the epidermal hypertrophy seen in the WT epidermis. However, this same reasoning would then also predict epidermal hypertrophy in the KO skin, because these keratinocytes have lost a key signaling intermediate, Smad3, in addition to the receptor loss. The significantly smaller acanthotic response in the KO epidermis instead suggests that TGF-β has altered other responses to TGF-β that may remain intact in the WT cells, or that KO keratinocytes may also have an altered response pattern to factors other than TGF-β1, including mitogens such as keratinocyte growth factor. The control of keratinocyte proliferation is complex and has been shown to be dependent on a double-paracrine interaction with fibroblasts in which interleukin-1 secreted by keratinocytes induces fibroblasts to secrete keratinocyte growth factor that then promotes keratinocyte proliferation. Deletion of Smad3 could alter this signaling loop, or, alternatively, TGF-β may affect keratinocyte growth factor expression by fibroblasts. An example of this exists in limbal fibroblasts where TGF-β treatment markedly up-regulates protein levels of keratinocyte growth factor despite down-regulation of its transcript levels.

The high levels of receptor expression in the dermis of both WT and KO skin after irradiation suggest that fibroblasts and inflammatory cells remain responsive to TGF-β signals and that the striking difference in the numbers of inflammatory cells present in the WT and KO dermis 6 weeks after irradiation likely reflects effects of loss of Smad3 and its role in recruitment of these cells. TGF-β is chemotactic for mast cells, and neutrophils. Of these, loss of Smad3 is known to impair the chemotactic response of both neutrophils and macrophages to TGF-β and the likelihood is that it may impair chemotactic effects of TGF-β on the other cells as well. Our results suggest, for example, that Smad3 may also function in the TGF-β-mediated chemotaxis of mast cells, because a twofold increase in these cells was found in the dermis of WT mice compared to KO mice after irradiation. Neutrophils, previously shown to require Smad3 for chemotaxis to TGF-β, show a threefold difference. In contrast, we observed a similar radiation-dependent fold-increase in the number of macrophages in WT and KO skin (7.7-fold and 6.5-fold, respectively), suggesting that signals activated by irradiation other than TGF-β are involved in recruitment of these cells. Additional studies will be needed to determine whether the decreased numbers of inflammatory cells in KO animals result primarily from impaired chemotaxis to TGF-β because of loss of Smad3 or whether the lesser amounts of TGF-β in the KO skin also contribute to this effect.

![Figure 5](https://example.com/figure5.png)

Figure 5. Survival of Smad3 WT (solid bars) and KO (open bars) keratinocytes (A) at 2 days and dermal fibroblasts (B) at 5 days after exposure to various doses of irradiation. Results are the average of three experiments.
Although we have not directly quantitated collagen levels, we hypothesize that KO mice will, at later times, exhibit a significantly reduced fibrotic response compared to WT mice after irradiation because the dermis of KO mice contains one-half the number of myofibroblasts as determined by α-smooth muscle actin staining and because those myofibroblasts are exposed to reduced levels of TGF-β. The fibrotic response is characterized by the transdifferentiation of fibroblasts into myofibroblasts that acquire contractile properties and secrete matrix proteins. This conversion, which is transitory in normal wound healing, does not resolve in chronic tissue fibrosis. Because TGF-β has been implicated in the fibroblast to myofibroblast transition, the reduced numbers of myofibroblasts seen in the KO skin may be a result of the lower levels of TGF-β expressed in these animals or alternatively, the transition may be directly dependent on Smad3. Growth inhibitory functions of TGF-β have been reported to be lost as fibroblasts differentiate to myofibroblasts, despite retention of receptor expression levels and unchanged expression levels of Smad proteins. Experiments using primary cultures of pig myofibroblasts suggest that there is a specific defect in nuclear translocation of Smad3 in these cells underlying their escape from TGF-β growth inhibition. Although the complex milieu of cytokines that the cells are exposed to in vitro makes it difficult to compare our results directly with these in vitro studies, our data showing fewer myofibroblasts in KO mice after irradiation suggest that Smad3 may participate in both the recruitment of fibroblasts to the site of injury and possibly also the differentiation of fibroblasts to myofibroblasts. In addition to the reduced numbers of myofibroblasts in KO dermis, the dependence of TGF-β-induced synthesis of collagens 1, 3, 5, and 6 on Smad3, also is strongly suggestive that loss of Smad3 will be protective against radiation fibrosis. Future studies will be aimed at evaluating such fibrotic endpoints directly, using later time points acquired from surviving members of irradiated cohorts.

It is not surprising that loss of Smad3 offers no protection against systemic effects of radiation caused by depletion of bone marrow progenitors or against radiation-induced death of cells in vitro. Rather, our prediction is that Smad3 effects will be seen in inflammatory and fibrotic components of the response of tissue to irradiation. Whether our results can be extended to other sites such as lung and intestine where fibrosis is common after radiation of patients, is not yet known. Regardless, our results provide some optimism that the ability to protect normal tissue during radiotherapy by interfering with specific signaling pathways could allow dose escalation for more effective tumor kill while decreasing the inflammatory response and the fibrotic sequelae that adversely affect a patient’s quality of life. Clearly the development of a selective Smad3 inhibitor could have important applications in radiation therapy.

Acknowledgment

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References


tation of type I collagen transcription in human skin fibroblasts by

27. Vindevoghel L, Lechleider RJ, Kuo A, de Caestecker MP, Uttilo, J, Rob-
erts AB, Mauvel A: Smad3/4-dependent transcriptional activation of the
human type V collagen gene (COL5A1) promoter by transforming

A: Smad3/AP-1 interactions control transcriptional responses to TGF-


30. Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE,
Anzano M, Greenwell-Wild T, Wahl SM, Deng C, Roberts AB: Mice lacking
Smad3 show accelerated wound healing and an impaired

31. Martin M, Lefaix J, Delanian S: TGF-beta1 and radiation fibrosis: a
master switch and a specific therapeutic target? Int J Radiat Oncol
Biol Phys 2000, 47:277–290

32. Martin M, Lefaix JL, Pinton P, Cretch F, Daburon F: Temporal mod-
ulation of TGF-beta 1 and beta-actin gene expression in pig skin and

33. Randall K, Coggle JE: Long-term expression of transforming growth
factor beta TGF-beta 1 in mouse skin after localized beta-irradiation. Int J

34. Yi ES, Bedoya A, Lee H, Chin E, Saunders W, Kim SJ, Danielpour D,
Remick DG, Yin S, Ulrich TR: Radiation-induced lung injury in vivo:
expression of transforming growth factor beta precedes fibrosis. In-

Changes in transforming growth factor-beta 1 gene expression and
immunoreactivity levels during development of chronic radiation en-

36. Kraft M, Oussoren Y, Stewart FA, Dorr W, Schultz-Hector S: Radiation-
induced changes in transforming growth factor beta and collagen
expression in the murine bladder wall and its correlation with bladder

37. Anscher MS, Cretch F, Daburon F: Temporal modulation of transforming
factor TGF-beta 1 in mouse skin in response to TGF-beta 1 stimulation.

38. Cuscela D, Coffin D, Lupton GP, Cook JA, Krishna MC, Bonner RF,
Mitchell JB: Protection from radiation-induced alopecia with topical ap-
2:273–278

and their increase by bone marrow transplantation. Blood 1978,
52:447–452

40. Flanders KC, Thompson NL, Cissel DS, Van Obberghen-Schilling E,
Baker CC, Kass ME, Ellingsworth LR, Roberts AB, Sporn MB: Trans-
forming growth factor-beta 1: histochemochemical localization with antibod-

41. Flanders KC, Leducque G, Engels S, Cissel DS, Roberts AB, Kondaiah
P, Lafyatis R, Sporn MB, Unsicker K: Localization and actions of trans-
forming growth factor-betas in the embryonic nervous system.

42. Dlugosz AA, Glick AB, Tennenbaum T, Weinberg WC, Yuspa SH:
Isolation and utilization of epidermal keratinocytes for oncogene re-

43. Lichti U, Weinberg WC, Goodman L, Ledbetter S, Dooley T, Morgan
D, Yuspa SH: In vivo regulation of murine hair growth: insights from
grafting defined cell pools developed in nude mice. J Invest Dermatol
1993, 101:S124–S129

44. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G: Transforming
growth factor-beta 1 induces alpha-smooth muscle actin expression in
granulation tissue myofibroblasts and in quiescent and growing

45. Serini G, Bochaton-Piallat ML, Ropraz P, Geinoz A, Borsi L, Zardi L,
Gabbiani G: The fibronectin domain ED-A is crucial for myofibroblas-
tic phenotype induction by transforming growth factor-beta1. J Cell