JCI The Journal of Clinical Investigation

α3β1 integrin–controlled Smad7 regulates reepithelialization during wound healing in mice

Louise E. Reynolds, … , C. Michael DiPersio, Kairbaan M. Hodivala-Dilke

J Clin Invest. 2008;118(3):965-974. https://doi.org/10.1172/JCI33538.

Research Article **Dermatology**

Effective reepithelialization after injury is essential for correct wound healing. The upregulation of keratinocyte α3β1 integrin during reepithelialization suggests that this adhesion molecule is involved in wound healing; however, its precise role in this process is unknown. We have shown here that retarded reepithelialization in *Itga3*$^{-/-}$ mouse skin wounds is due predominantly to repressed TGF-β1–mediated responses. Specifically, expression of the inhibitor of TGF-β1–signaling Smad7 was elevated in *Itga3*$^{-/-}$ keratinocytes. Indeed, in vivo blockade of Smad7 increased the rate of reepithelialization in *Itga3*$^{-/-}$ and WT wounds to similar levels. Our data therefore indicate that the function of α3β1 integrin as a mediator of keratinocyte migration is not essential for reepithelialization but suggest instead that α3β1 integrin has a major new in vivo role as an inhibitor of Smad7 during wound healing. Moreover, our study may identify a previously undocumented function for Smad7 as a regulator of reepithelialization in vivo and implicates Smad7 as a potential novel target for the treatment of cutaneous wounds.

α3β1 integrin–controlled Smad7 regulates reepithelialization during wound healing in mice

Louise E. Reynolds,[1] Francesco J. Conti,[2] Rita Silva,[1] Stephen D. Robinson,[1] Vandana Iyer,[3] Rob Rudling,[4] Barbara Cross,[4] Emma Nye,[5] Ian R. Hart,[1] C. Michael DiPersio,[3] and Kairbaan M. Hodivala-Dilke[1]

[1]Centre for Tumour Biology, Institute of Cancer Research and Cancer Research UK Clinical Centre, Barts and The London, Queen Mary's School of Medicine and Dentistry, John Vane Science Centre, London, United Kingdom. [2]The Scripps Research Institute, La Jolla, California, USA. [3]Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York, USA. [4]London Research Institute, Cancer Research UK, Hertfordshire, United Kingdom. [5]Experimental Pathology Laboratory, London Research Institute, Cancer Research UK, London, United Kingdom.

Effective reepithelialization after injury is essential for correct wound healing. The upregulation of keratinocyte α3β1 integrin during reepithelialization suggests that this adhesion molecule is involved in wound healing; however, its precise role in this process is unknown. We have shown here that retarded reepithelialization in *Itga3*$^{-/-}$ mouse skin wounds is due predominantly to repressed TGF-β1–mediated responses. Specifically, expression of the inhibitor of TGF-β1–signaling Smad7 was elevated in *Itga3*$^{-/-}$ keratinocytes. Indeed, in vivo blockade of Smad7 increased the rate of reepithelialization in *Itga3*$^{-/-}$ and WT wounds to similar levels. Our data therefore indicate that the function of α3β1 integrin as a mediator of keratinocyte migration is not essential for reepithelialization but suggest instead that α3β1 integrin has a major new in vivo role as an inhibitor of Smad7 during wound healing. Moreover, our study may identify a previously undocumented function for Smad7 as a regulator of reepithelialization in vivo and implicates Smad7 as a potential novel target for the treatment of cutaneous wounds.

Introduction

The failure of wounds to heal is an important clinical problem not only after surgery or injury but also in patients with chronic conditions, such as diabetes. The lack of wound repair in such individuals can result in major disability or even death. Understanding the precise molecular mechanisms of wound repair regulation in vivo is important in developing effective strategies to accelerate correct healing of the skin. Repair of adult skin is a complex process that is thought to be influenced both by growth factors, such as TGF-β1, and by adhesion molecules, such as integrins. However, little is known about the coordinated effects of these molecules in wound repair in vivo.

TGF-β1 is expressed highly during wound healing and plays critical functions in initiating and modulating reepithelialization (1, 2). TGF-β1 elicits its effects by interacting first with constitutively active TGF-β type II receptors (TGF-βRII) which, in turn, recruit and activate TGF-β type I receptors (TGF-βRI). Formation of the TGF-β receptor complexes leads to the subsequent phosphorylation and heterodimerization of intracellular downstream effectors, receptors Smad2 and Smad3. The phosphorylated Smad2/3 complex associates with Smad4 (3), which translocates to the nucleus, binds DNA, initiates gene transcription, and results in changes in cell behavior. TGF-β signaling is regulated negatively by inhibitory Smads such as Smad7 (4, 5). One mechanism by which Smad7 can function is by competing with Smad2 and Smad3 for association with the TGF-β receptor complex. Elucidating the mechanism of Smad7 regulation in vivo is important therefore for the development of new methods to control TGF-β1–mediated responses. Although the mechanisms underlying TGF-β–mediated signaling have been examined in vitro (6, 7), much less is known about their regulation during wound healing in vivo (8, 9).

Importantly, the roles of other families of molecules that may be required for the regulation of TGF-β1–mediated responses in vivo often are underestimated. Expression levels of most keratinocyte integrins are elevated transiently in the epidermis during reepithelialization (10, 11), suggesting their involvement in this process (12–14). Integrin α3β1 is overexpressed in basal and suprabasal layers of the epidermis during reepithelialization, but the nature of its function during wound healing is controversial (10, 11). In vitro studies indicate that α3β1 is essential for keratinocyte migration on laminin 5 (Lm 5) (15, 16), and these data have fueled the preconception that α3β1 integrin is essential for keratinocyte migration during reepithelialization in vivo. Contrary to these data, α3β1 binding to unprocessed Lm 5, which is deposited early in reepithelialization, has been shown either to retard or promote migration (17–19). Moreover, experiments using different anti-α3β1 function–blocking antibodies have revealed that they can either inhibit long-term keratinocyte-substrate adhesion (16) or, conversely, increase migration on fibronectin (Fn) and collagen type IV (Coll IV) (20, 21). Furthermore, genetic ablation of α3 integrin is associated with enhanced keratinocyte migration on Fn and Coll IV without changes in the expression of other keratinocyte integrins, suggesting that α3β1 can act as a transdominant inhibitor of Fn- and Coll IV–specific integrin receptors (15). Taken together, these observations suggest that the functions of α3β1 integrin during wound healing in vivo are likely to be more com-

Nonstandard abbreviations used: Coll IV, collagen type IV; Ent, entactin; Fn, fibronectin; Lm 5, laminin 5; PCNA, proliferating cell nuclear antigen; TGF-βRI, TGF-β type I receptor(s).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **118**:965–974 (2008). doi:10.1172/JCI33538.

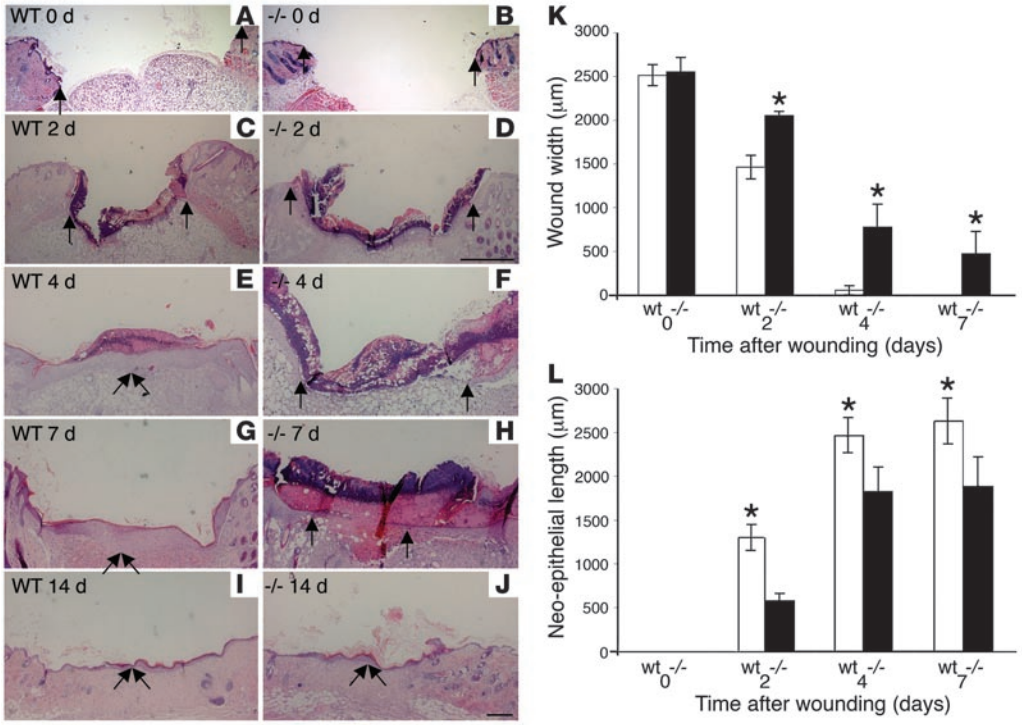

Figure 1
Reepithelialization is impaired in *Itga3*$^{-/-}$ skin. Midline sections of WT (**A**, **C**, **E**, **G**, and **I**) and *Itga3*$^{-/-}$ (–/–) (**B**, **D**, **F**, **H**, and **J**) wounds were stained with H&E for examination of reepithelialization at 0 (**A** and **B**), 2 (**C** and **D**), 4 (**E** and **F**), 7 (**G** and **H**), and 14 days (**I** and **J**) after injury. (**K**) Quantitation of wound widths. (**L**) Quantitation of neoepithelial length. Results represent the mean ± SEM; $n = 11$/time point/genotype. *$P \leq 0.05$. Arrows represent the edges of migrating epithelial lips. Scale bars: 750 μm (**A**–**D**); 375 μm (**E**–**J**).

plex than envisaged initially and raise questions about the possible role of this integrin in wound healing. Using *Itga3*$^{-/-}$ skin, we tested the role of α3β1 integrin during wound healing. Here, we report that although the reepithelialization rate is impaired in *Itga3*$^{-/-}$ wounds relative to controls, the migratory function of α3β1 is secondary to its role as a negative regulator of Smad7. Our data identify Smad7 as a novel regulator of reepithelialization, making it a new target for the treatment of cutaneous wounds.

Results

Impaired reepithelialization in Itga3$^{-/-}$ *skin*. We determined whether α3 integrin deficiency had any effect on wound repair. Full-thickness 3-mm punch-biopsy wounds were made in *Itga3*$^{-/-}$ and WT skin grafts and the rate of wound closure assessed by analysis of H&E-stained sections taken at 0, 2, 4, 7, and 14 days after injury (Figure 1, A–J). Wound healing in WT skin was complete by 7 days after injury. In contrast, wound repair was impaired significantly in *Itga3*$^{-/-}$ skin, and complete closure occurred several days after closure of WT wounds. Reepithelialization (measured by wound widths and lengths of neoepithelium) and wound contraction (measured by distance between wound margins) are important processes that can control the overall rate of repair. We observed that the wound widths were larger and neoepithelial lengths shorter in *Itga3*$^{-/-}$ healing skin when compared with controls (Figure 1, K and L), while no significant differences in the distance between wound margins were found between genotypes (data not shown). These results indicate that the reduced rate of wound healing observed in *Itga3*$^{-/-}$ skin was not due to altered wound contraction but was a result of defective reepithelialization.

Reepithelialization is dependent on several cellular changes, including altered deposition of ECM components, upregulation of keratinocyte integrins, increased keratinocyte proliferation, and decreased apoptosis. Our results show that, apart from the expected loss of α3 integrin in *Itga3*$^{-/-}$ wounds, the distribution patterns of α3, β1, α5, α6, β4, and α2 integrin subunits (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI33538DS1; and data not shown) and ECM components Fn, entactin (Ent), and Lm 5 (Supplemental Figure 1B) were similar to those observed in WT controls. We have shown previously that α3β1 is required for the maintenance rather than the deposition of Lm 5 (22, 23). Thus, the apparently normal Lm 5 expression pattern observed during the early stages of reepithelialization in *Itga3*$^{-/-}$ wounds most likely reflects newly deposited Lm 5. In addition, no differences in levels of proliferation and apoptosis were detected between WT and *Itga3*$^{-/-}$ keratinocytes during reepithelialization (data not shown). Taken together, these results suggest that delayed *Itga3*$^{-/-}$ reepithelialization is due to reduced epithelial migration, which is independent of altered patterns of ECM deposition, integrin expression, keratinocyte proliferation, or apoptosis.

Repressed TGF-β1–mediated migration in Itga3$^{-/-}$ *cells*. To evaluate the mechanism responsible for the impaired reepithelialization in *Itga3*$^{-/-}$ wounds, we analyzed keratinocyte migration in an in vitro wound model. Confluent monolayers of WT and *Itga3*$^{-/-}$ keratinocytes were scratched and cell migration across the scratch measured after 24 hours. Surprisingly, the rates of scratch closure for *Itga3*$^{-/-}$ and WT keratinocytes in vitro were similar (Figure 2A). Several experiments have demonstrated that integrin signaling can alter cell migration in response to growth factors (24, 25). During wound healing, the concentration of TGF-β1 in WT skin increases to approximately 1 ng/ml (26) and is thought to regulate reepithelialization (1, 27, 28). Therefore, we hypothesized that TGF-β1 may differentially affect *Itga3*$^{-/-}$ and WT keratinocyte scratch closure. In contrast to untreated controls, 1 ng/ml TGF-β1 stimulated closure of WT keratinocytes but inhibited migration of *Itga3*$^{-/-}$ keratinocytes in vitro (Figure 2A). In similar experi-

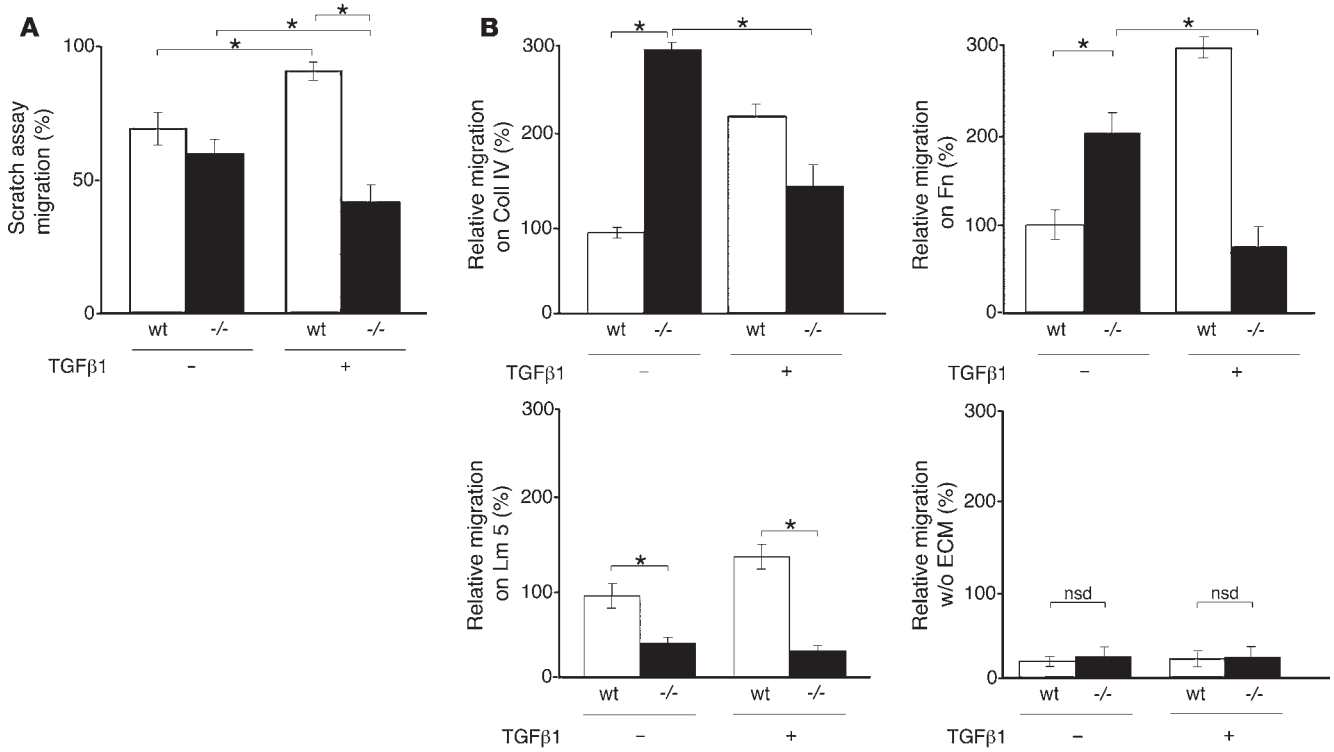

Figure 2

TGF-β1–mediated migration is reduced in *Itga3*$^{-/-}$ keratinocytes. Confluent cultures of WT or *Itga3*$^{-/-}$ keratinocytes were scratched, either in the absence (–) or presence (+) of 1 ng/ml TGF-β1, and changes in the rate of scratch closure were observed 24 hours after wounding (**A**). Results in **A** represent the mean scratch width at 24 hours relative to the scratch width at 0 hours (%) ± SEM; $n = 3$ separate samples/genotype/treatment. Single-cell migration of WT and *Itga3*$^{-/-}$ keratinocytes in the absence or presence of TGF-β1 on Coll IV, Fn, Lm 5, or no ECM (**B**). Results in **B** represent the mean migration of single cells (±SEM) relative to untreated WT controls. $n = 3$ experiments. $^*P < 0.05$.

ments, treatment with EGF, another growth factor implicated in reepithelialization, enhanced the migration of keratinocytes from both genotypes (data not shown).

During reepithelialization in vivo, keratinocytes migrate on newly deposited ECM. To determine whether specific migratory responses to individual ECMs were affected by TGF-β1, we analyzed single-cell migration of both genotypes on Fn, Coll IV, and Lm 5 (Figure 2B). Single-cell migration of *Itga3*$^{-/-}$ keratinocytes on Fn or Coll IV in the absence of TGF-β1 was enhanced significantly when compared with WT controls. These results corroborate our previous findings showing that activity of α2β1 and α5β1 integrins is upregulated in *Itga3*$^{-/-}$ keratinocytes (15). In the presence of TGF-β1, WT cell migration on Fn and Coll IV was enhanced significantly when compared with untreated WT controls. Conversely, addition of TGF-β1 to *Itga3*$^{-/-}$ cells reduced migration on Fn and Coll IV significantly, but migration on Lm 5 was not affected. On uncoated glass, the migration of WT and *Itga3*$^{-/-}$ cells, either in the presence or absence of TGF-β1, was barely detectable, and no differences in migration rates were observed between genotypes. Importantly, integrin expression levels were identical for both genotypes before and after TGF-β1 treatment, except for the absence of α3 subunits in *Itga3*$^{-/-}$ keratinocytes (15) (data not shown), suggesting that α3 deficiency does not result in compensatory responses by the overexpression of other keratinocyte integrins.

Taken together, these results suggest that α3β1 integrin plays a role in promoting responses to TGF-β1 and that the delayed reepithelialization observed in *Itga3*$^{-/-}$ wounds may be attributable to reduced TGF-β1–mediated migration.

α3β1 integrin regulates Smad7. Since Smad7 is known to be an inhibitory regulator of TGF-β1–mediated signaling (4, 5), we examined the effect of α3 integrin deficiency on Smad7 expression. Immunohistochemical analysis showed that in both genotypes, Smad7 and phospho-Smad2 were undetectable in uninjured epidermis (data not shown). In contrast, quantitation of Smad7 immunostaining showed significantly increased expression in *Itga3*$^{-/-}$ 2-day wounded epidermis when compared with WT 2-day wounds (Figure 3A). In addition, nuclear phospho-Smad2 staining, an indicator of positive TGF-β1 signaling, was decreased significantly in *Itga3*$^{-/-}$ epidermis when compared with controls (Figure 3B), suggesting that elevated Smad7 corresponded with inhibited TGF-β1–mediated signaling and delayed reepithelialization in vivo.

Western blot analysis of keratinocytes grown in vitro also revealed a significant increase in Smad7 levels in *Itga3*$^{-/-}$ cells when compared with WT (Figure 3C), and transduction of human α3 integrin into *Itga3*$^{-/-}$ keratinocytes restored Smad7 expression down to WT levels (data not shown). In contrast, transcript levels of Smad7 were not affected by α3 integrin (Supplemental Figure 2). Although we did not detect any reproducible differences in total levels of Smad2 and Smad3 between genotypes in the presence or absence of TGF-β1, TGF-β1–treated *Itga3*$^{-/-}$ keratinocytes expressed significantly less phospho-Smad2 and phospho-Smad3

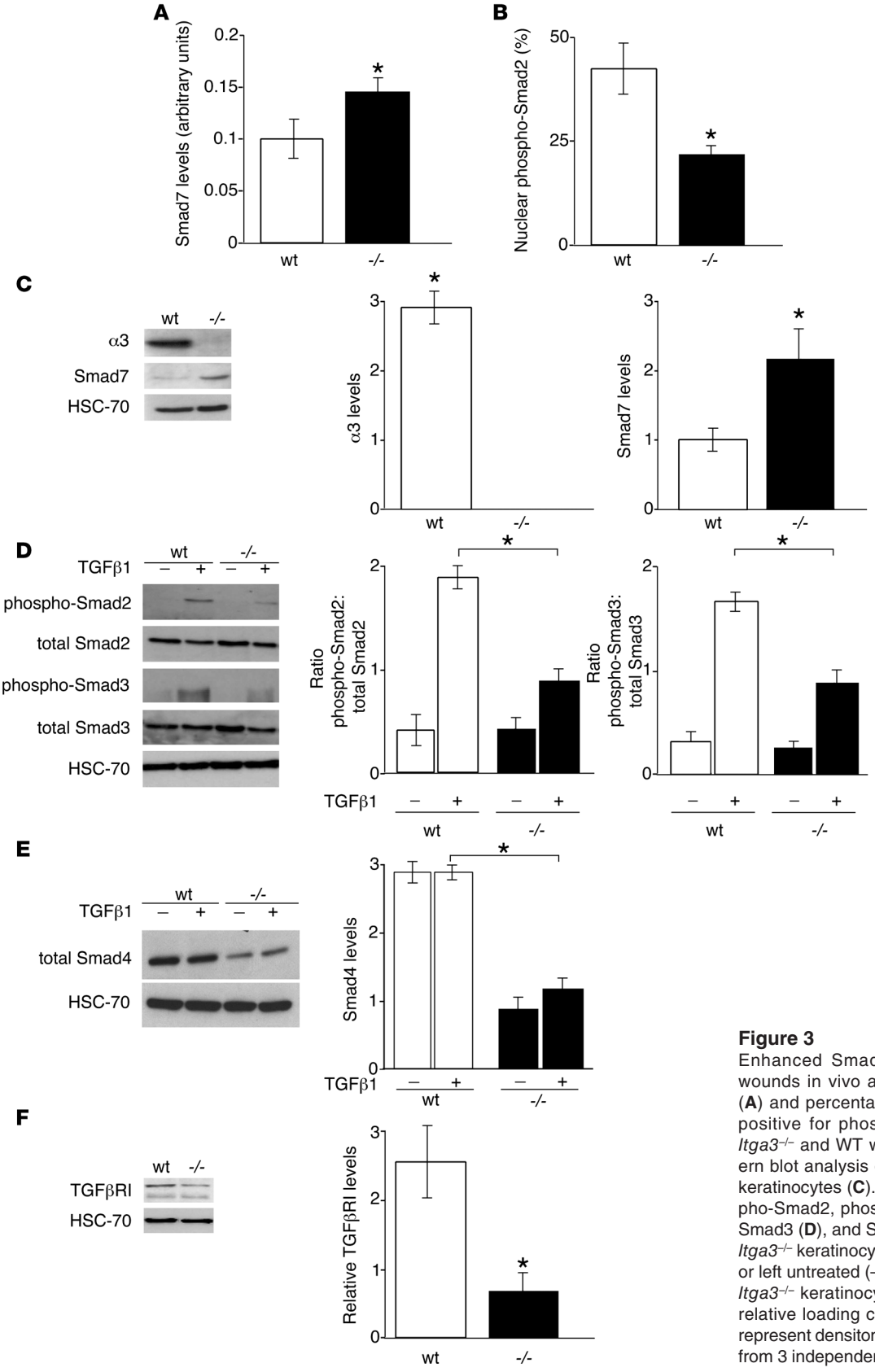

Figure 3
Enhanced Smad7 expression in *Itga3*$^{-/-}$ wounds in vivo and in vitro. Smad7 levels (**A**) and percentages of keratinocyte nuclei positive for phospho-Smad2 (**B**) in 2-day *Itga3*$^{-/-}$ and WT wounded epidermis. Western blot analysis of Smad7 protein levels in keratinocytes (**C**). Western blotting for phospho-Smad2, phospho-Smad3, total Smad2, Smad3 (**D**), and Smad4 levels (**E**) in WT and *Itga3*$^{-/-}$ keratinocytes treated with TGF-β1 (+) or left untreated (–). TGF-βRI levels in WT or *Itga3*$^{-/-}$ keratinocytes (**F**). HSC-70 provides relative loading controls. Bar charts in **C**–**F** represent densitometry results (mean ± SEM) from 3 independent experiments. *$P < 0.05$.

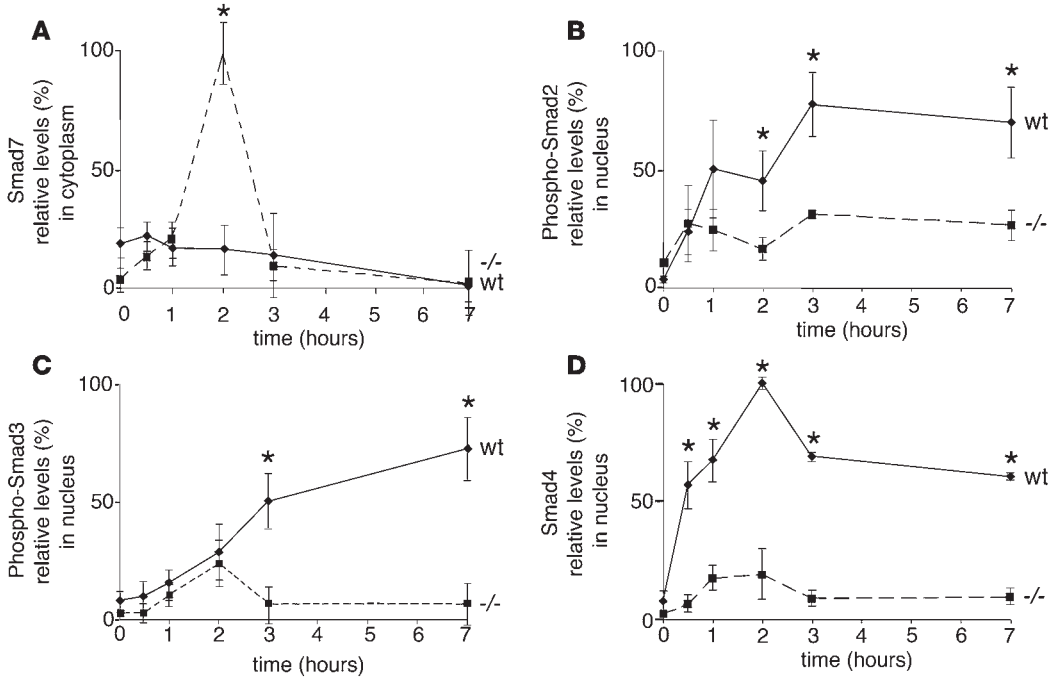

Figure 4
Cytoplasmic translocation of Smad7 is increased in *Itga3*$^{-/-}$ keratinocytes. WT or *Itga3*$^{-/-}$ keratinocytes were treated with TGF-β1 for 0, 0.5, 1, 2, 3, or 7 hours, and cytoplasmic or nuclear extracts were analyzed by Western blotting for Smad7 (**A**), phospho-Smad2 (**B**), phospho-Smad3 (**C**), and Smad4 (**D**). Graphs represent mean (±SEM) densitometry results relative to mean HSC-70 (for cytoplasmic extracts, **A**) or PCNA (for nuclear extracts, **B**–**D**) loading control levels. n = 3–4 independent experiments. Solid line, WT; dashed line, *Itga3*$^{-/-}$. $^{*}P < 0.05$.

when compared with similarly treated WT keratinocytes (Figure 3D). Examination of Smad4 revealed significantly lower levels in *Itga3*$^{-/-}$ keratinocytes when compared with WT, although the presence of TGF-β1 had no effect on Smad4 levels in either genotype (Figure 3E). Moreover, the expression levels of TGF-βRI were decreased in TGF-β1–treated *Itga3*$^{-/-}$ keratinocytes (Figure 3F). Since TGF-βRII is thought to be constitutively active, it is not surprising that the levels of this receptor were not changed between genotypes (data not shown). These results indicate that α3β1 integrin is a regulator of Smads both in vivo and in vitro.

Smad7 inhibits TGF-β1–mediated signaling by its transient translocation from the nucleus to the cytoplasm, where it blocks TGF-β receptor binding to phospho-Smad2/3 and, as a consequence, nuclear translocation of phospho-Smad2 and phospho-Smad3 is reduced. By Western blot analysis, we showed that nuclear-to-cytoplasmic translocation of Smad7 in *Itga3*$^{-/-}$ keratinocytes peaked 2 hours after TGF-β1 stimulation, at significantly higher levels than those observed in WT controls, and then returned back to WT levels (Figure 4A). Furthermore, cytoplasmic-to-nuclear translocation of phospho-Smad2, phospho-Smad3, and Smad4 was reduced significantly in *Itga3*$^{-/-}$ keratinocytes when compared with WT controls (Figure 4, B–D). We next tested the promoter activities of Smad7, Smad2, and Smad3/4 by transfection of WT and *Itga3*$^{-/-}$ keratinocytes with either pS7.5, ARE, or CAGA Smad-luciferase promoter constructs, respectively. Smad7 promoter activity was increased significantly in *Itga3*$^{-/-}$ keratinocytes in response to TGF-β1 (Figure 5A) whereas, in contrast, the promoter activities of Smad2, Smad3, and Smad4 were all decreased significantly when compared with WT controls (Figure 5, B and C). Together, these data show that decreased Smad7 protein levels correspond with decreased nuclear-to-cytoplasmic translocation and promoter activity of this Smad in *Itga3*$^{-/-}$ keratinocytes when compared with WT controls.

Smad7 is a new target for the treatment of wounds. To determine directly whether the increased Smad7 expression in *Itga3*$^{-/-}$ keratinocytes was responsible for their decreased TGF-β1–mediated migration, we depleted Smad7 by either Smad7-specific siRNA or Smad7-antisense oligonucleotide transfection in WT and *Itga3*$^{-/-}$ keratinocytes and tested relative TGF-β1–mediated scratch closure in vitro. Smad7 depletion by siRNA or by Smad7-antisense oligonucleotide transfection reduced Smad7 levels by 60%–90% and enhanced *Itga3*$^{-/-}$ keratinocyte migration significantly (2- to 3-fold) when compared with random siRNA- or Smad7-sense–transfected controls (Figure 6, A and B).

To understand the mechanism by which Smad7 regulated keratinocyte migration, we tested the effect of Smad7 inhibition, using antisense oligonucleotide transfection, on WT and *Itga3*$^{-/-}$ cell migration on specific ECM substrates in combination with TGF-β1. Smad7-antisense transfection increased *Itga3*$^{-/-}$ keratinocyte migration on Fn to a higher level than observed for WT keratinocytes (Figure 6C). Such differences were not observed on Coll IV or Lm 5 (data not shown). These data suggest that Smad7 specifically inhibits the integrins involved in migration on Fn and thus regulates reepithelialization.

Last, we determined whether inhibition of Smad7 expression would alter reepithelialization rates in wounded skin in vivo with the intention of testing Smad7 as a possible novel target for the treatment of wound repair. Pluronic gels containing Smad7-sense or -antisense oligonucleotides were applied to wounded WT or *Itga3*$^{-/-}$ skin (Figure 6, D–H). Smad7-antisense oligonucleotide treatment decreased Smad7 protein levels (Supplemental Figure 3A) and increased phospho-Smad2 nuclear localization in the *Itga3*$^{-/-}$ neoepidermis, enhancing significantly *Itga3*$^{-/-}$ wound reepithelialization. Smad7 levels were decreased in WT neoepidermis but, surprisingly, no change in nuclear phospho-Smad2 levels was observed (Supplemental Figure 3B). These responses did not involve Smad7-dependent changes in keratinocyte proliferation (Supplemental Figure 3C). Furthermore, Smad7-antisense oligonucleotide treatment of *Itga3*$^{-/-}$ keratinocytes enhanced TGF-βRI levels (Supplemental Figure 3D). Importantly, Smad7-antisense oligonucleotide treatment restored *Itga3*$^{-/-}$ reepithelialization to

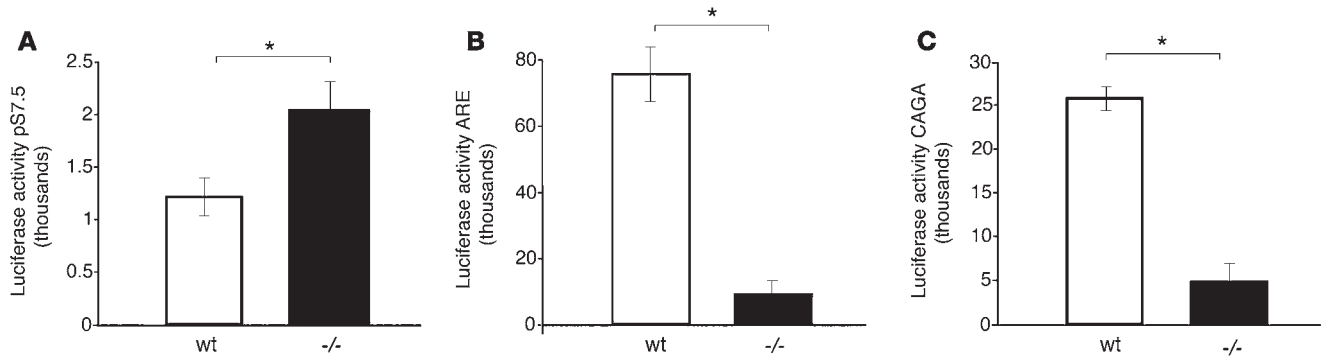

Figure 5
Elevated Smad7 promoter activity in *Itga3*$^{-/-}$ keratinocytes. WT and *Itga3*$^{-/-}$ keratinocytes were transfected with Smad-luciferase promoter constructs pS7.5 to test for Smad7 promoter activity (**A**), ARE/FAST1 to test for Smad2 promoter activity (**B**), and CAGA to test for Smad3/4 promoter activity (**C**). Graphs represent mean ± SEM luciferase activity. n = 6–12 separate samples in triplicate/treatment. *$P < 0.04$.

WT levels, demonstrating that the migratory function of α3β1 integrin is not required for reepithelialization. Treatment with the controls, Smad7-sense oligonucleotide, or PBS had no effect on wound closure in either genotype. These data indicate that the increased Smad7 expression in *Itga3*$^{-/-}$ keratinocytes is responsible for inhibiting keratinocyte migration during wound healing in vivo.

Discussion

Our data indicate that the migratory function of keratinocyte α3β1 is not crucial for wound healing in vivo but rather identifies a predominant new role for α3β1 during this process as an inhibitor of Smad7. These experiments demonstrate a previously unknown function for Smad7 as a regulator of reepithelialization in vivo and highlight a new and effective method to treat wounds by inhibiting Smad7.

Prior to our study, α3β1 integrin has been thought to be important in keratinocyte adhesion in vitro (15, 18, 22, 29, 30); however, the precise role of this integrin in keratinocyte migration, especially in vivo, has been controversial. For example, several lines of data suggest that α3β1 plays additional roles: (a) α3β1 integrin expression during wound healing is increased mostly in suprabasal keratinocytes (10, 11, 31), where it is unlikely to mediate adhesion to the ECM; (b) Lm 5, the major ligand for α3β1 in the epidermis, is deposited at the wound edge in an unprocessed form (18, 19, 30) and, since α3β1 has been reported to bind inefficiently to unprocessed Lm 5 (17, 20), it is possible that this integrin does not have major Lm 5–adhesive functions during in vivo wound healing; (c) Ent, another proposed ligand for α3β1, is not detectable at the wound edge and is present only in the basement membrane of resting epidermis; (d) α3β1 can act as a transdominant inhibitor of other integrins, namely α5β1, the Fn receptor, and α2β1, a Coll IV receptor, since inhibition of α3β1 by function-blocking antibodies or genetic ablation (15) can enhance migration on Fn and Coll IV. Taken together, these observations, although not conclusive, suggest that in the absence of α3β1, wound repair could proceed normally or even be enhanced.

Our data indicate that α3 deficiency per se is not sufficient to inhibit the rate of keratinocyte migration in the absence of growth factors in in vitro scratch closure assays or, after Smad7 inhibition, during reepithelialization in vivo. This is not to say that α3β1 integrin is not required for migration on Lm 5 but does highlight the need to reassess the role of this integrin in vivo. Our data suggest that α3β1 is a negative regulator of Smad7 protein, and we speculate that binding of α3β1 integrin to Lm or other α3 ligands during wound healing may suppress Smad7 expression. Smad7 levels are elevated in *Itga3*$^{-/-}$ keratinocytes both in vivo and in vitro with a corresponding decrease in TGF-βRI levels and a reduction in Smad2 and Smad3 phosphorylation, Smad4 protein levels, and Smad2/4 nuclear translocation. Our results are supported by observations in K5-Smad7 transgenic mice, where overexpression of Smad7 in the epidermis antagonizes Smad2 phosphorylation without affecting total Smad2 levels and also inhibits TGF-βRI protein expression levels (32).

The changes in Smad2 and Smad3 activity in *Itga3*$^{-/-}$ keratinocytes could partly explain the aberrant reepithelialization in *Itga3*$^{-/-}$ skin since these Smads have been implicated in wound healing previously (8, 33). For example, our results are consistent with observations of impaired wound healing in Smad3 heterozygous mice (8).

We have reported previously that α3β1 can act as a transdominant inhibitor of both α2β1 and α5β1 (15). Since here we show that inhibition of Smad7 specifically increases *Itga3*$^{-/-}$ keratinocyte migration on Fn but not other tested matrices including Coll, it is tempting to speculate that Smad7 regulates reepithelialization by inhibiting the function of the Fn receptor α5β1 integrin, i.e., treatment of wounds with Smad7 inhibitors may relieve the transdominant function of α3β1 over α5β1, thereby enhancing the overall rate of reepithelialization. The lack of an effect of Smad7 inhibitors on Coll-mediated migration likely reflects a Smad7-independent mechanism by which α3β1 regulates TGF-β1–stimulated migration. Although we do not exclude other Smad7-independent mechanisms, we show for what we believe is the first time that Smad7, under the control of the integrin α3β1, is a pivotal regulator of wound healing and that inhibition of Smad7 expression accelerates wound healing in vivo.

Despite the extensive studies elucidating the role of TGF-β1 in modulating integrin function (34–38), the effect of β1 integrins on TGF-β1–mediated Smad signaling has received very little attention. Impaired wound closure and reduced keratinocyte migration without changes in epidermal cell proliferation have been observed in mice lacking β1 integrin in the epidermis (14), suggesting a critical role for β1 integrins in wound healing. However, the effect of this integrin subunit or its partner α-subunits on the regulation of TGF-β1–mediated responses in keratinocytes has not been examined. The overexpression of α6 integrin subunits in suprabasal keratinocytes is associated with reduced Smad2 phosphorylation (39), but wound

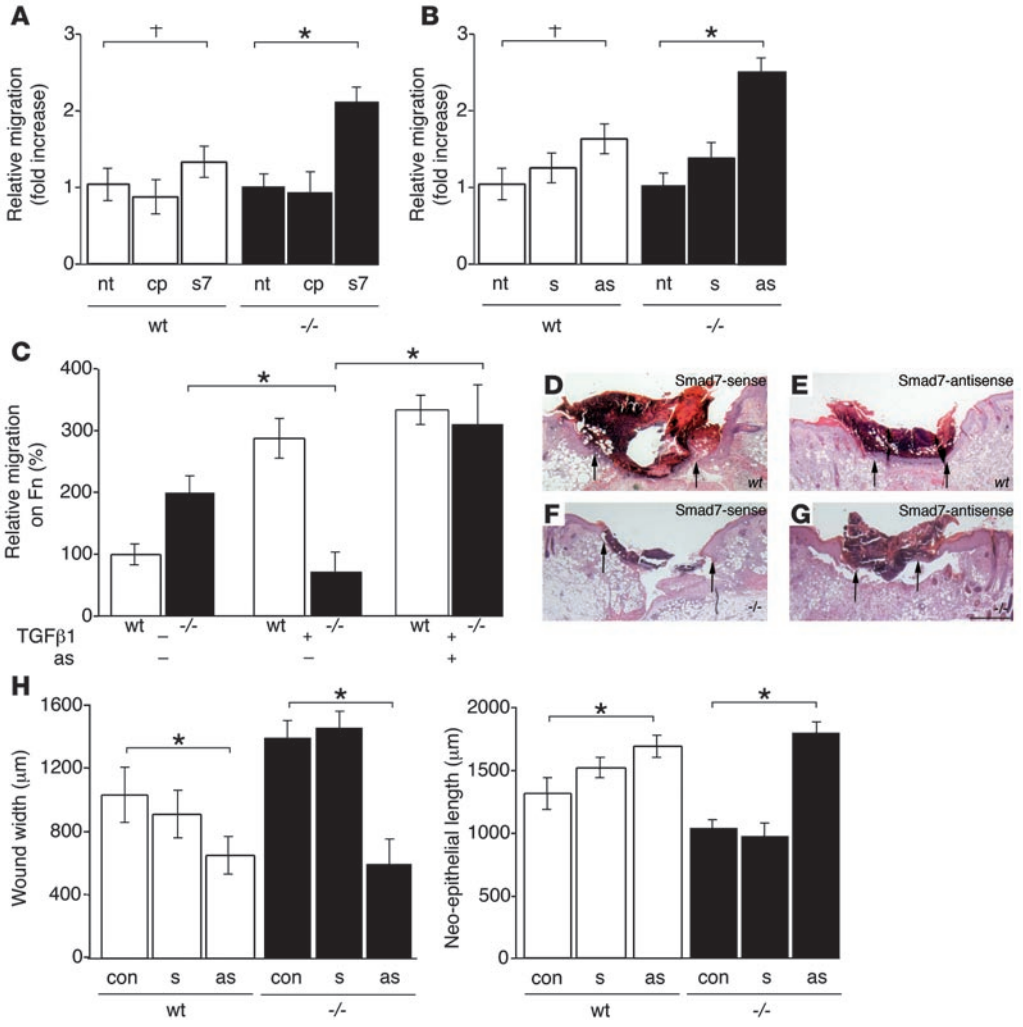

Figure 6
Inhibition of Smad7 restores migration of *Itga3*$^{-/-}$ keratinocytes in vitro and reepithelialization in vivo. Analysis of scratch-wound closure in WT or *Itga3*$^{-/-}$ keratinocyte cultures untransfected (nt) or transfected with control pool (cp) or Smad7 (s7) siRNAi (**A**) or sense (s) or antisense (as) Smad7 oligonucleotides (**B**). Results in **A** and **B** represent the relative migration (fold increase) ± SEM. $n = 30$–35 data points/treatment. Single-cell migration of WT and *Itga3*$^{-/-}$ keratinocytes on Fn in the presence (+) or absence (–) of TGF-β1 and Smad7-antisense oligonucleotides (**C**). Results in **C** represent relative migration to untreated WT controls. H&E-stained sections of WT (**D** and **E**) and *Itga3*$^{-/-}$ (**F** and **G**) full-thickness wounds treated with either Smad7-sense (**D** and **F**) or Smad7-antisense oligonucleotides (**E** and **G**) at 3 days after injury. Scale bar: 750 μm. (**H**) Quantitation of wound widths and neoepithelial lip lengths of WT and *Itga3*$^{-/-}$ wounds treated with either PBS control (con), Smad7-sense, or Smad7-antisense oligonucleotides 3 days after injury. *$P < 0.009$ for all experiments; †$P < 0.4$. Arrows represent the edges of migrating epithelial lips.

healing in these mice has not been tested. We reported previously that β3 integrin deficiency in dermal fibroblasts enhanced TGF-β1–mediated responses with a corresponding increase in wound closure rate, suggesting that β3 integrin can act as a negative regulator of TGF-β1 signaling (26). Our present data provide evidence for a previously unidentified role for α3β1 integrin in the negative regulation of Smad7, both in vivo and in vitro. Considering the essential role of TGF-β1 signaling in wound healing, we demonstrate that one of the functions of integrins, other than as adhesion molecules, is to act as regulators of this pathway in vivo.

Usually cutaneous wound healing does not require medical treatment. However, intervention to aid the rate of wound healing could be critical in circumstances such as extensive injury or in ulcerative processes. By elucidating the role of α3β1 integrin in reepithelialization as a negative regulator of Smad7, we have identified what we believe is a novel and major function for Smad7 as a repressor of wound repair. Our data offer a new clinical approach to accelerating wound healing by inhibiting keratinocyte Smad7.

Methods

Antibodies. Rabbit antisera to the α3 integrin (clone 8-4) and β1 integrin (clone 210-H) subunits were prepared as described (40). The antibody to α6 integrin was from Chemicon International. The antibodies to β4 integrin and Smad2 were purchased from BD Biosciences. The antibody specific to α2 integrin was a kind gift from Beate Eckes (University of Cologne, Cologne, Germany). Rabbit antisera to Fn was kindly provided by R.O. Hynes (MIT, Cambridge, Massachusetts, USA). The anti–Lm 5 polyclonal antibody was a generous gift from Peter Marinkovich (Stanford University School of Medicine, Stanford, California, USA). Antibodies to Ent and TGF-βRII were purchased from Upstate Biotechnology. Antibodies to TGF-βRI and HSC-70 were purchased from Autogen Bioclear. The antibody to Smad3 was purchased from Zymed Laboratories Inc. Anti-Smad7 was from Imgenex. Proliferating cell nuclear antigen (PCNA) antibody was obtained from Cancer Research UK. Anti–phospho-Smad2 and anti–phospho-Smad3 were purchased from Cell Signaling Technologies for Western blot analysis or were kind gifts from Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) for immunohistochemistry. All the FITC- and HRP-conjugated secondary antibodies were purchased from Biosource International.

Immunostaining. Immunostaining of 6-μm cryosections was carried out as follows: sections were fixed in 4% formaldehyde for 20 minutes at room temperature or, for α2 integrin, fixed in ice-cold acetone for 8 minutes. For detection of Ent, Lm 5, and α3, β1, α6, β4, and α2 integrins, sections were blocked for 1 hour at room temperature. For detection of Fn, sections were blocked in 1% Fn-free FCS for 1 hour at room temperature. Sections then were incubated in primary antibody for 40 minutes at room temperature, washed in PBS, incubated with biotin-conjugated secondary

antibody for 40 minutes, washed in PBS, and then incubated with FITC-conjugated streptavidin for 40 minutes. Finally, sections were washed and mounted in Gelvatol supplemented with DABCO antifading agent. Representative fields were photographed using a Hamamatsu Digital Camera (Improvision) on a Zeiss Telaval microscope (Zeiss). For α2 integrin immunostaining, images were taken using a Zeiss Axiovert 200M confocal microscope (Zeiss). Comparisons of these immunostaining patterns were carried out in a double-blind fashion on 4–5 samples per genotype; each experiment was repeated at least 3 times.

Skin-grafting and wound-healing experiments. Skin grafting was performed as described previously (22, 23, 41). At 20–30 days after grafting, mice were anesthetized with halothane, skin grafts were shaved and cleaned with 70% alcohol, and full-thickness wounds were made using a 3-mm biopsy punch. Wounded tissue was collected at 0, 2, 4, 7, and 14 days after injury and processed as described below.

Wound processing and morphological analysis. Skin grafts were shaved and excised and wounds were bisected along the anterior-posterior axis of the skin and either embedded in OCT compound (Thermo Life Sciences) and snap-frozen or fixed in 4% formaldehyde, then embedded in paraffin. For morphological analysis, sections from the center of paraffin-embedded wounds were stained with H&E. Quantitation was carried out in a double-blind fashion on 11 separate graft samples from each wound time point for both genotypes. The wound width was quantitated by measuring the distance between epithelial lips across the wound bed. Neoepithelial length was calculated by adding together the lengths of the newly formed epithelium from the tip of the reepithelialization lip to the site of the first hair follicle at the wound margin. Measurements were done on a Zeiss Telaval microscope using OpenLab analysis software (Improvision).

Immunohistochemistry for Smads. Paraffin-embedded 2-, 4-, and 7-day WT and *Itga3*$^{-/-}$ wounds were immunostained for Smad7 or phospho-Smad2. In brief, sections were dewaxed in xylene and rehydrated in decreasing ethanol concentrations; this was followed by antigen retrieval by microwaving slides with 0.01 M citrate buffer. Sections were blocked for 30 minutes and incubated overnight with either Smad7 or phospho-Smad2 antibody. Sections were then washed and incubated with a biotinylated secondary antibody (DAKO). Further washes were performed, and the sections were incubated with Vectastain ABC reagent (Vector Laboratories). DAB (Sigma-Aldrich) was added to the sections and incubated until optimal color development was observed. Sections were counterstained with hematoxylin, dehydrated, and mounted with NeoMount. Histological analysis for Smad7 levels was performed with a light microscope (Eclipse E1000; Nikon) and digital imaging software (Lucia V.5).

Keratinocyte cultures. Immortalized WT and *Itga3*$^{-/-}$ mouse keratinocytes were prepared as described previously (42). Keratinocytes were isolated from mouse skin and maintained at 33°C in keratinocyte growth medium. Keratinocyte growth medium consisted of low Ca^{2+} FAD (Cancer Research UK), 10% FCS (chelated), 10^{-10} M cholera toxin, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin (all from Sigma-Aldrich), 10 ng/ml EGF (Life Technologies), and 10 U/ml IFN-γ (Peprotech). Immortalized keratinocytes were used for all experiments.

In vitro scratch closure assay. WT and *Itga3*$^{-/-}$ immortalized keratinocytes were grown to 80%–90% confluency in 6-well plates (allowing the cells to produce their own ECM) and serum starved overnight. Scratches were made across each well with a plastic pipette tip. Without changing the medium, each well was treated with either 1 ng/ml TGF-β1 (Peprotech) or left untreated. To determine the rate of in vitro scratch closure, the scratch width was measured at 0 and 24 hours after wounding, using a grid inserted in the microscope eyepiece of a Zeiss Telaval microscope. The rate of scratch closure was calculated as the difference between the wound width at 24 hours and the original scratch width at time 0 hours. Scratch width measurements at 24 hours are given as the percentage of the original scratch width at 0 hours for each scratch (as in Figure 2A) or as a fold change in migration relative to nontreated controls for both genotypes (as in Figure 6, A and B).

Single-cell migration assays. Single-cell migration assays were performed according to the method of Albrecht-Buehler (43). In brief, WT and *Itga3*$^{-/-}$ immortalized keratinocyte cultures were washed in PBS and cells fed with keratinocyte serum-free medium (Gibco; BRL) containing supplements and 1% FCS for 24 hours. Cells then were trypsinized and washed in serum-containing medium, and single-cell suspensions were resuspended in keratinocyte serum-free medium (containing 1% FCS) and allowed to adhere and migrate on colloidal gold lawns coated with 50 μg/ml of either Fn, Coll IV, Lm 5, or no ECM in the presence or absence of 1 ng/ml TGF-β1 for 16 hours. Cells then were fixed and area translocated by single cells quantified in a double-blind fashion using an OpenLab quantitation program on a Zeiss Telaval microscope. Migration assays were repeated 3 times, and a minimum of 50 cells/test were assessed. Migration rates are presented as average migration of cells relative to migration of WT keratinocytes on Fn.

Western blot analysis. Keratinocytes were grown to 70% confluency, serum-starved overnight in Opti-MEM plus 1% FCS followed by overnight stimulation with 1 ng/ml TGF-β1, and lysed with RIPA buffer. Equal amounts of protein from each genotype were electrophoresed on 8%–12% SDS-polyacrylamide gels, followed by transfer onto nitrocellulose membrane (Hybond-ECL; Amersham Biosciences). Membranes were blocked for 30 minutes in either 3% or 5% milk-PBS-Tween (0.05%) followed by incubation with antibodies directed to either TGF-βRI, TGF-βRII, Smad2, phospho-Smad2, Smad3, phospho-Smad3, Smad4, Smad7, α3 integrin, or HSC-70 for 1 hour at room temperature or overnight at 4°C. Membranes were washed 3 times in PBS-Tween (0.05%) followed by incubation with an HRP-conjugated secondary antibody for 1 hour at room temperature and washed and incubated with ECL reagents (Amersham Biosciences). Chemiluminescence was detected by exposure to X-Omat AR films (Kodak). Densitometric readings of band intensities were obtained using Lab Images software for 3–5 samples per test. HSC-70 was used as a loading control.

Smad nuclear translocation. WT and *Itga3*$^{-/-}$ immortalized keratinocytes were pretreated for 30 minutes with 20 μg/ml of cycloheximide to prevent further protein synthesis. Cells were treated with 2 ng/ml TGF-β1 for 0, 0.5, 1, 2, 3, and 7 hours. At the final time point, cells were washed in PBS, and 500 μl of ice-cold hypotonic lysis buffer was added to each culture dish. Cells were scraped off, and the nuclei and cellular debris were pelleted at 500 *g*. The supernatant (cytoplasmic fraction) was collected and stored at –80°C. The pellet was resuspended in 100 μl of nuclear extraction buffer and rotated end-on-end at 4°C for 3 hours. Finally, the lysates were cleared of debris by centrifugation and processed by SDS-PAGE. PCNA antibody was used to normalize for nuclear lysates, and HSC-70 was used to normalize for cytoplasmic lysates. Densitometric readings of band intensities were carried out using Lab Images software.

Transfections and transcriptional reporter assays. WT and *Itga3*$^{-/-}$ immortalized keratinocytes were seeded in 24-well plates and transfected with luciferase reporter constructs ARE and FAST1 (Smad2), CAGA (Smad3/4) (gift from A. Roberts) and pS7.5 (Smad7) (gift from E. Bottinger) and *Renilla* using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated with starvation medium (Opti-MEM plus1% FCS) for 24 hours and then were either left untreated or were treated with TGF-β1 (5 ng/ml) for 8 hours. Luciferase and *Renilla* (to determine transfection efficiency) activities in transfected cells were determined using the dual luciferase assay kit from Promega.

Smad7 RT-PCR. RNA was extracted from 80% confluent WT and *Itga3*$^{-/-}$ immortalized keratinocytes using the TRIzol method (Invitrogen) after treatment with or without 1 ng/ml TGF-β1. RNA was reverse transcribed

using the Superscript III First-Strand Synthesis Kit (Invitrogen). The primers used to identify Smad7 were as follows: 5′-TGGCATACTGGGAGGAGAAGAC-3′; 5′-CAGCCCTTCACAAAGCTGAT-3′. The primers produced a 400-bp product. DNA was amplified in a 25 μl reaction under the following conditions: 96°C, 60 seconds; 62°C, 60 seconds; 72°C, 60 seconds; repeated for 35 cycles. Products were resolved on 1.8% agarose gels. Actin was used as a control for loading.

Smad7 RNAi transfection and scratch-wound closure. 40% confluent WT and *Itga3*$^{-/-}$ immortalized keratinocytes were transfected with siRNA against Smad7 or a scrambled control pool (Dharmacon) for 72 hours, according to the manufacturer's instructions. At 80%–90% confluency, keratinocytes were serum starved overnight with Opti-MEM containing 1% chelated FCS, then scratched as described above, in the presence of 1 ng/ml TGF-β1. Cell migration was calculated as the difference between the wound width at 24 hours and the original scratch width at time 0 hours.

Smad7 oligonucleotide transfection in vitro. 40% confluent *Itga3*$^{-/-}$ immortalized keratinocyte cultures were transfected with Smad7 sense and antisense oligonucleotides at a final concentration of 4 μg/ml using Lipofectamine Plus Reagent (Invitrogen). Details of the Smad7 nucleotides have been reported previously (44). In brief, phosphorothioate single-stranded oligonucleotide matching the region 107–128 (5′-GCTGCGGGGAGAAGGGGCGAC-3′) of the human Smad7 complementary DNA sequence was synthesized in the sense and antisense orientation. For scratch-wound assays, 90% confluent *Itga3*$^{-/-}$ keratinocyte cultures were washed in PBS, starved overnight with Opti-MEM containing 1% chelated FCS, and treated with 1 ng/ml TGF-β1 for 24 hours. Scratches were made using a plastic pipette tip and the rate of cell migration determined after 48 hours as described above. For single-cell migration assays, cells were transfected with Smad7 sense and antisense oligonucleotides for 24 hours, grown to 70% confluency, and used in single-cell scratch assays as described.

Inhibition of Smad7 in vivo. 100 ng of either sense or antisense phosphorothioate single-stranded oligonucleotide to human Smad7 or PBS was mixed in Pluronic gel F127 (Sigma-Aldrich) and administered to WT or *Itga3*$^{-/-}$ skin grafts immediately after wounding (45). At 3 days after injury, mice were killed and wounded skin harvested for histological analysis.

Statistics. Statistical significance was calculated using a 2-tailed Student's *t* test for all experiments. $P < 0.05$ was considered statistically significant.

Animal regulations. All animals were used in accordance with United Kingdom Home Office regulations and after ethical review and approval by the Cancer Research UK ethics committee.

Acknowledgments

We thank Caroline Hill, Simon Broad, Gareth Inman, Richard Grose, John Marshall, Amanda Daley, Andrew Reynolds, David Becker, Fiona Watt, and David Owens for their advice and criticism throughout the work; Jess Grulinger for her technical assistance; and George Elias and colleagues for help with histology.

Received for publication August 7, 2007, and accepted in revised form November 28, 2007.

Address correspondence to: Kairbaan M. Hodivala-Dilke, Centre for Tumour Biology, Institute of Cancer Research and Cancer Research UK Clinical Centre, Barts and The London, Queen Mary's School of Medicine and Dentistry, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, United Kingdom. Phone: 44-207-014-0406; Fax: 44-207-014-0401; E-mail: Kairbaan.Hodivala-Dilke@cancer.org.uk.

Louise E. Reynolds and Francesco J. Conti contributed equally to this work.

1. Singer, A.J., and Clark, R.A. 1999. Cutaneous wound healing. *N. Engl. J. Med.* **341**:738–746.
2. Ashcroft, G.S., et al. 1997. Estrogen accelerates cutaneous wound healing associated with an increase in TGF-beta1 levels. *Nat. Med.* **3**:1209–1215.
3. Massague, J. 2000. How cells read TGF-beta signals. *Nat. Rev. Mol. Cell Biol.* **1**:169–178.
4. Nakao, A., et al. 1997. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature.* **389**:631–635.
5. Hayashi, H., et al. 1997. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell.* **89**:1165–1173.
6. Dennler, S., et al. 1998. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.* **17**:3091–3100.
7. Moustakas, A., Souchelnytskyi, S., and Heldin, C.H. 2001. Smad regulation in TGF-beta signal transduction. *J. Cell Sci.* **114**:4359–4369.
8. Ashcroft, G.S., et al. 1999. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat. Cell Biol.* **1**:260–266.
9. Tan, N.S., et al. 2004. Essential role of Smad3 in the inhibition of inflammation-induced PPARbeta/delta expression. *EMBO J.* **23**:4211–4221.
10. Cavani, A., et al. 1993. Distinctive integrin expression in the newly forming epidermis during wound healing in humans. *J. Invest. Dermatol.* **101**:600–604.
11. Hertle, M.D., Kubler, M.D., Leigh, I.M., and Watt, F.M. 1992. Aberrant integrin expression during epidermal wound healing and in psoriatic epidermis. *J. Clin. Invest.* **89**:1892–1901.
12. Huang, X., Griffiths, M., Wu, J., Farese, R.V., Jr., and Sheppard, D. 2000. Normal development, wound healing, and adenovirus susceptibility in beta5-deficient mice. *Mol. Cell. Biol.* **20**:755–759.
13. Hakkinen, L., et al. 2004. Increased expression of beta6-integrin in skin leads to spontaneous development of chronic wounds. *Am. J. Pathol.* **164**:229–242.
14. Grose, R., et al. 2002. A crucial role of beta 1 integrins for keratinocyte migration in vitro and during cutaneous wound repair. *Development.* **129**:2303–2315.
15. Hodivala-Dilke, K.M., DiPersio, C.M., Kreidberg, J.A., and Hynes, R.O. 1998. Novel roles for alpha3beta1 integrin as a regulator of cytoskeletal assembly and as a trans-dominant inhibitor of integrin receptor function in mouse keratinocytes. *J. Cell Biol.* **142**:1357–1369.
16. Carter, W.G., Kaur, P., Gil, S.G., Gahr, P.J., and Wayner, E.A. 1990. Distinct functions for integrins alpha 3 beta 1 in focal adhesions and alpha 6 beta 4/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.* **111**:3141–3154.
17. Decline, F., and Rousselle, P. 2001. Keratinocyte migration requires alpha2beta1 integrin-mediated interaction with the laminin 5 gamma2 chain. *J. Cell Sci.* **114**:811–823.
18. Goldfinger, L.E., et al. 1999. The alpha3 laminin subunit, alpha6beta4 and alpha3beta1 integrin coordinately regulate wound healing in cultured epithelial cells and in the skin. *J. Cell Sci.* **112**:2615–2629.
19. Frank, D.E., and Carter, W.G. 2004. Laminin 5 deposition regulates keratinocyte polarization and persistent migration. *J. Cell Sci.* **117**:1351–1363.
20. Decline, F., et al. 2003. Keratinocyte motility induced by TGF-beta1 is accompanied by dramatic changes in cellular interactions with laminin 5. *Cell Motil. Cytoskeleton.* **54**:64–80.
21. Kim, J.P., Zhang, K., Kramer, R.H., Schall, T.J., and Woodley, D.T. 1992. Integrin receptors and RGD sequences in human keratinocyte migration: unique anti-migratory function of alpha 3 beta 1 epiligrin receptor. *J. Invest. Dermatol.* **98**:764–770.
22. DiPersio, C.M., et al. 1997. alpha3beta1 Integrin is required for normal development of the epidermal basement membrane. *J. Cell Biol.* **137**:729–742.
23. Conti, F.J., Rudling, R.J., Robson, A., and Hodivala-Dilke, K.M. 2003. alpha3beta1-integrin regulates hair follicle but not interfollicular morphogenesis in adult epidermis. *J. Cell Sci.* **116**:2737–2747.
24. Eliceiri, B.P. 2001. Integrin and growth factor receptor crosstalk. *Circ. Res.* **89**:1104–1110.
25. Del Pozo, M.A., et al. 2002. Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. *Nat. Cell Biol.* **4**:232–239.
26. Reynolds, L.E., et al. 2005. Accelerated re-epithelialization in beta3-integrin-deficient- mice is associated with enhanced TGF-beta1 signaling. *Nat. Med.* **11**:167–174.
27. Garlick, J.A., and Taichman, L.B. 1994. Effect of TGF-beta 1 on re-epithelialization of human keratinocytes in vitro: an organotypic model. *J. Invest. Dermatol.* **103**:554–559.
28. Shah, M., et al. 1999. Role of elevated plasma transforming growth factor-beta 1 levels in wound healing. *Am. J. Pathol.* **154**:1115–1124.
29. Zhang, K., and Kramer, R.H. 1996. Laminin 5 deposition promotes keratinocyte motility. *Exp. Cell Res.* **227**:309–322.
30. Nguyen, B.P., Gil, S.G., and Carter, W.G. 2000. Deposition of laminin 5 by keratinocytes regulates integrin adhesion and signaling. *J. Biol. Chem.* **275**:31896–31907.

31. Grinnell, F. 1992. Wound repair, keratinocyte activation and integrin modulation. *J. Cell Sci.* **101**:1–5.
32. He, W., et al. 2002. Overexpression of Smad7 results in severe pathological alterations in multiple epithelial tissues. *EMBO J.* **21**:2580–2590.
33. Arany, P.R., et al. 2006. Smad3 deficiency alters key structural elements of the extracellular matrix and mechanotransduction of wound closure. *Proc. Natl. Acad. Sci. U. S. A.* **103**:9250–9255.
34. Zambruno, G., et al. 1995. Transforming growth factor-beta 1 modulates beta 1 and beta 5 integrin receptors and induces the de novo expression of the alpha v beta 6 heterodimer in normal human keratinocytes: implications for wound healing. *J. Cell Biol.* **129**:853–865.
35. Heino, J., Ignotz, R.A., Hemler, M.E., Crouse, C., and Massague, J. 1989. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. *J. Biol. Chem.* **264**:380–388.
36. Wahl, S.M., Allen, J.B., Weeks, B.S., Wong, H.L., and Klotman, P.E. 1993. Transforming growth factor beta enhances integrin expression and type IV collagenase secretion in human monocytes. *Proc. Natl. Acad. Sci. U. S. A.* **90**:4577–4581.
37. Bartolome, R.A., Sanz-Rodriguez, F., Robledo, M.M., Hidalgo, A., and Teixido, J. 2003. Rapid upregulation of alpha4 integrin-mediated leukocyte adhesion by transforming growth factor-beta1. *Mol. Biol. Cell.* **14**:54–66.
38. Gailit, J., Welch, M.P., and Clark, R.A. 1994. TGF-beta 1 stimulates expression of keratinocyte integrins during re-epithelialization of cutaneous wounds. *J. Invest. Dermatol.* **103**:221–227.
39. Owens, D.M., Romero, M.R., Gardner, C., and Watt, F.M. 2003. Suprabasal alpha6beta4 integrin expression in epidermis results in enhanced tumourigenesis and disruption of TGFbeta signalling. *J. Cell Sci.* **116**:3783–3791.
40. Marcantonio, E.E., Guan, J.L., Trevithick, J.E., and Hynes, R.O. 1990. Mapping of the functional determinants of the integrin beta 1 cytoplasmic domain by site-directed mutagenesis. *Cell Regul.* **1**:597–604.
41. Kreidberg, J.A., et al. 1996. Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development.* **122**:3537–3547.
42. DiPersio, C.M., Shao, M., Di Costanzo, L., Kreidberg, J.A., and Hynes, R.O. 2000. Mouse keratinocytes immortalized with large T antigen acquire alpha3beta1 integrin-dependent secretion of MMP-9/gelatinase B. *J. Cell Sci.* **113**:2909–2921.
43. Albrecht-Buehler, G. 1977. The phagokinetic tracks of 3T3 cells. *Cell.* **11**:395–404.
44. Monteleone, G., et al. 2001. Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *J. Clin. Invest.* **108**:601–609.
45. Becker, D.L., et al. 1999. Roles for alpha 1 connexin in morphogenesis of chick embryos revealed using a novel antisense approach. *Dev. Genet.* **24**:33–42.