

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

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Effective reepithelialization after injury is essential for correct wound healing. The upregulation of keratinocyte  $\alpha 3\beta 1$  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- $\beta 1$ -mediated responses. Specifically, expression of the inhibitor of TGF- $\beta 1$ -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  $\alpha 3\beta 1$  integrin as a mediator of keratinocyte migration is not essential for reepithelialization but suggest instead that  $\alpha 3\beta 1$  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- $\beta$ 1, and by adhesion molecules, such as integrins. However, little is known about the coordinated effects of these molecules in wound repair in vivo.

TGF- $\beta$ 1 is expressed highly during wound healing and plays critical functions in initiating and modulating reepithelialization (1, 2). TGF- $\beta$ 1 elicits its effects by interacting first with constitutively active TGF- $\beta$  type II receptors (TGF- $\beta$ RII) which, in turn, recruit and activate TGF- $\beta$  type I receptors (TGF- $\beta$ RI). Formation of the TGF- $\beta$  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- $\beta$  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

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

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with the TGF- $\beta$  receptor complex. Elucidating the mechanism of Smad7 regulation in vivo is important therefore for the development of new methods to control TGF- $\beta$ 1-mediated responses. Although the mechanisms underlying TGF- $\beta$ -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  $\alpha 3\beta 1$  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,  $\alpha 3\beta 1$  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  $\alpha 3$ integrin is associated with enhanced keratinocyte migration on Fn and Coll IV without changes in the expression of other keratinocyte integrins, suggesting that  $\alpha 3\beta 1$  can act as a transdominant inhibitor of Fn- and Coll IV-specific integrin receptors (15). Taken together, these observations suggest that the functions of  $\alpha 3\beta 1$ integrin during wound healing in vivo are likely to be more com-



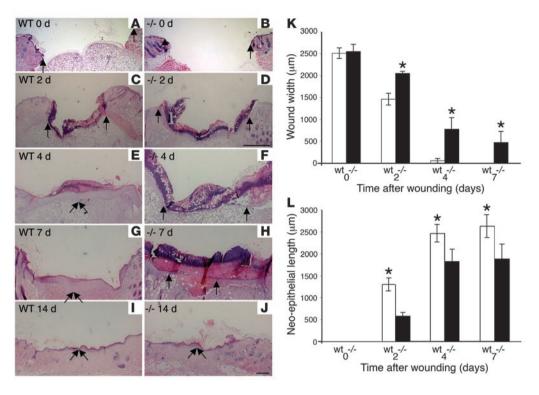


Figure 1 Reepithelialization is impaired in Itga3-/- skin. Midline sections of WT (A, C, E, G, and I) and Itga3-/- (-/-) (B, D, F, H, and J) wounds were stained with H&E for examination of reepithelialization at 0 (A and **B**), 2 (**C** and **D**), 4 (**E** and **F**), 7 (G and H), and 14 days (I and J) after injury. (K) Quantitation of wound widths. (L) Quantitation of neoepithelial length. Results represent the mean  $\pm$  SEM; n = 11/time point/ genotype. \* $P \le 0.05$ . 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  $\alpha 3\beta 1$  integrin during wound healing. Here, we report that although the reepithelialization rate is impaired in Itga3-/- wounds relative to controls, the migratory function of  $\alpha 3\beta 1$  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*<sup>-/-</sup> *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*<sup>-/-</sup> 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*<sup>-/-</sup> wounds, the distribution patterns of  $\alpha 3$ ,  $\beta 1$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 4$ , and  $\alpha 2$  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  $\alpha 3\beta 1$  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*<sup>-/-</sup> 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*<sup>-/-</sup> 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<sup>-/-</sup> cells. To evaluate the mechanism responsible for the impaired reepithelialization in *Itga3*<sup>-/-</sup> 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*<sup>-/-</sup> 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- $\beta 1$  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*<sup>-/-</sup> 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-



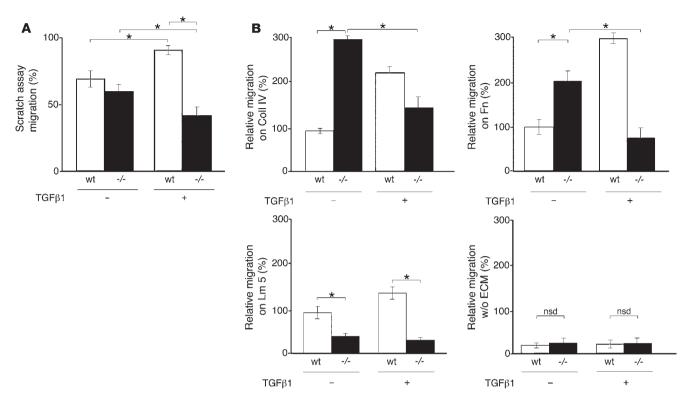


Figure 2 TGF- $\beta$ 1—mediated migration is reduced in *Itga3*<sup>-/-</sup> keratinocytes. Confluent cultures of WT or *Itga3*<sup>-/-</sup> keratinocytes were scratched, either in the absence (–) or presence (+) of 1 ng/ml TGF- $\beta$ 1, and changes in the rate of scratch closure were observed 24 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*<sup>-/-</sup> keratinocytes in the absence or presence of TGF- $\beta$ 1 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*<sup>-/-</sup> 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  $\alpha 2\beta 1$  and  $\alpha 5\beta 1$  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  $\alpha 3$  subunits in *Itga3*<sup>-/-</sup> keratinocytes (15) (data not shown), suggesting that  $\alpha 3$  deficiency does not result in compensatory responses by the overexpression of other keratinocyte integrins.

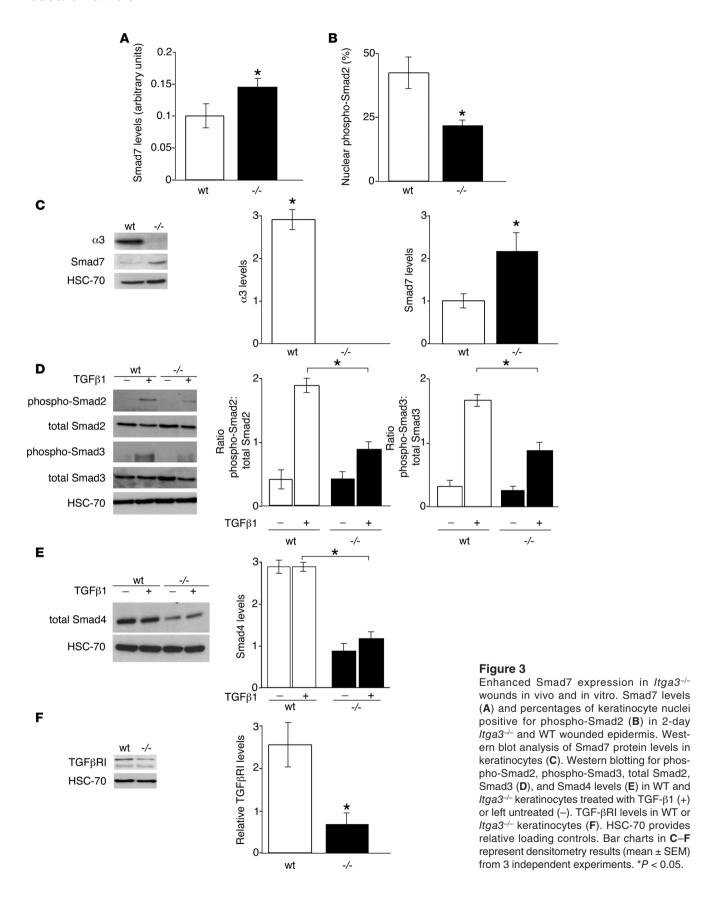
Taken together, these results suggest that  $\alpha 3\beta 1$  integrin plays a role in promoting responses to TGF- $\beta 1$  and that the delayed reepi-

thelialization observed in  $Itga3^{-/-}$  wounds may be attributable to reduced TGF- $\beta1$ -mediated migration.

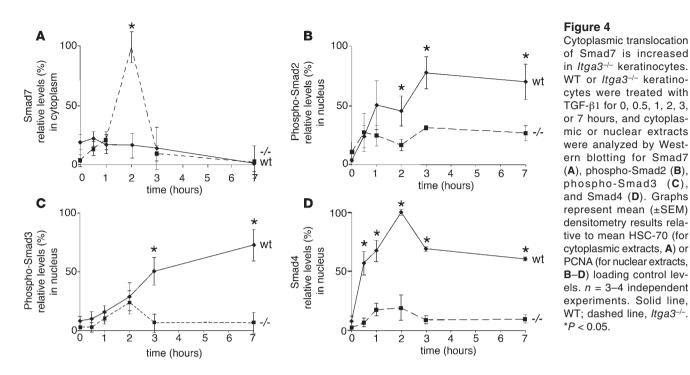
 $\alpha 3\beta 1$  integrin regulates Smad7. Since Smad7 is known to be an inhibitory regulator of TGF- $\beta 1$ -mediated signaling (4, 5), we examined the effect of  $\alpha 3$  integrin deficiency on Smad7 expression. Immunohistochemical analysis showed that in both genotypes, Smad7 and phospho-Smad2 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- $\beta 1$  signaling, was decreased significantly in  $Itga3^{-/-}$  epidermis when compared with controls (Figure 3B), suggesting that elevated Smad7 corresponded with inhibited TGF- $\beta 1$ -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  $\alpha 3$  integrin into  $Itga3^{-/-}$  keratinocytes restored Smad7 expression down to WT levels (data not shown). In contrast, transcript levels of Smad7 were not affected by  $\alpha 3$  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- $\beta 1$ , TGF- $\beta 1$ -treated  $Itga3^{-/-}$  keratinocytes expressed significantly less phospho-Smad2 and phospho-Smad3









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)

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*<sup>-/-</sup> 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  $\alpha 3\beta 1$  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*<sup>-/-</sup> 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*<sup>-/-</sup> 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 nuclearto-cytoplasmic translocation and promoter activity of this Smad in *Itga3*<sup>-/-</sup> 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-\$1-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<sup>-/-</sup> 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*<sup>-/-</sup> cell migration on specific ECM substrates in combination with TGF-β1. Smad7-antisense transfection increased *Itga3*<sup>-/-</sup> 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



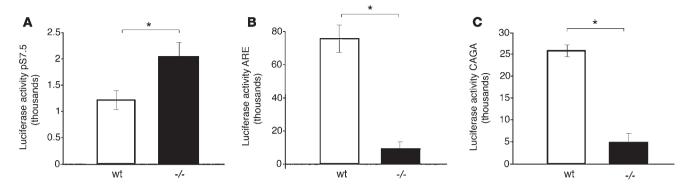


Figure 5
Elevated Smad7 promoter activity in *Itga3*<sup>-/-</sup> keratinocytes. WT and *Itga3*<sup>-/-</sup> keratinocytes were transfected with Smad-luciferase promoter constructs pS7.5 to test for Smad7 promoter activity (**A**), ARE/FAST1 to test for Smad2 promoter activity (**B**), and CAGA to 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  $\alpha 3\beta 1$  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  $\alpha 3\beta 1$  is not crucial for wound healing in vivo but rather identifies a predominant new role for  $\alpha 3\beta 1$  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  $\alpha 3\beta 1$  plays additional roles: (a)  $\alpha 3\beta 1$  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  $\alpha 3\beta 1$  in the epidermis, is deposited at the wound edge in an unprocessed form (18, 19, 30) and, since  $\alpha 3\beta 1$  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  $\alpha 3\beta 1$ , is not detectable at the wound edge and is present only in the basement membrane of resting epidermis; (d)  $\alpha 3\beta 1$  can act as a transdominant inhibitor of other integrins, namely  $\alpha 5\beta 1$ , the Fn receptor, and  $\alpha 2\beta 1$ , 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  $\alpha 3\beta 1$ , wound repair could proceed normally or even be enhanced.

Our data indicate that  $\alpha 3$  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  $\alpha 3\beta 1$  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

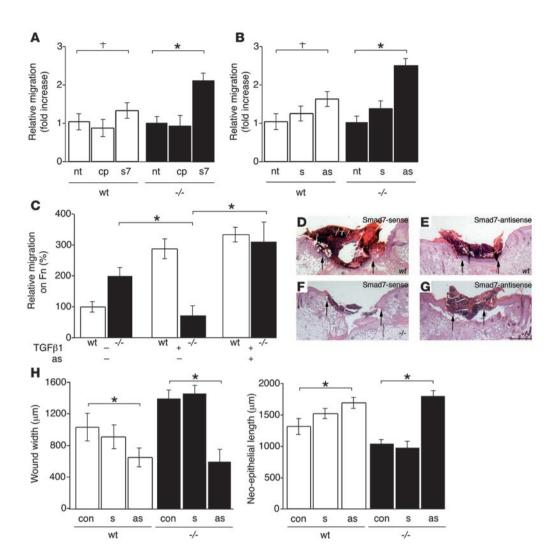
 $\alpha 3\beta 1$  is a negative regulator of Smad7 protein, and we speculate that binding of  $\alpha 3\beta 1$  integrin to Lm or other  $\alpha 3$  ligands during wound healing may suppress Smad7 expression. Smad7 levels are elevated in Itga3<sup>-/-</sup> keratinocytes both in vivo and in vitro with a corresponding decrease in TGF- $\beta$ RI 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- $\beta$ RI 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  $\alpha 3\beta 1$  can act as a transdominant inhibitor of both  $\alpha 2\beta 1$  and  $\alpha 5\beta 1$  (15). Since here we show that inhibition of Smad7 specifically increases *Itga3*<sup>-/-</sup> 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  $\alpha 3\beta 1$  over  $\alpha 5\beta 1$ , 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  $\alpha 3\beta 1$ , 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- $\beta1$  in modulating integrin function (34–38), the effect of  $\beta1$  integrins on TGF- $\beta1$ -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  $\beta1$  integrin in the epidermis (14), suggesting a critical role for  $\beta1$  integrins in wound healing. However, the effect of this integrin subunit or its partner  $\alpha$ -subunits on the regulation of TGF- $\beta1$ -mediated responses in keratinocytes has not been examined. The overexpression of  $\alpha6$  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 Smad7antisense 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 Smad7sense (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;  $^{\dagger}P$  < 0.4. Arrows represent the edges of migrating epithelial lips.

healing in these mice has not been tested. We reported previously that  $\beta 3$  integrin deficiency in dermal fibroblasts enhanced TGF- $\beta 1$ -mediated responses with a corresponding increase in wound closure rate, suggesting that  $\beta 3$  integrin can act as a negative regulator of TGF- $\beta 1$  signaling (26). Our present data provide evidence for a previously unidentified role for  $\alpha 3\beta 1$  integrin in the negative regulation of Smad7, both in vivo and in vitro. Considering the essential role of TGF- $\beta 1$  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  $\alpha 3\beta 1$  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  $\alpha 3$  integrin (clone 8-4) and  $\beta 1$  integrin (clone 210-H) subunits were prepared as described (40). The antibody to  $\alpha 6$  integrin was from Chemicon International. The antibodies to  $\beta 4$  inte-

grin and Smad2 were purchased from BD Biosciences. The antibody specific to  $\alpha 2$  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- $\beta$ RII were purchased from Upstate Biotechnology. Antibodies to TGF- $\beta$ RI 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  $\alpha 2$  integrin, fixed in ice-cold acetone for 8 minutes. For detection of Ent, Lm 5, and  $\alpha 3$ ,  $\beta 1$ ,  $\alpha 6$ ,  $\beta 4$ , and  $\alpha 2$  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

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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  $\alpha$ 2 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 doubleblind 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 Ca²+ 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- $\gamma$  (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- $\beta$ 1 (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, a3 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  $\mu g/ml$  of cycloheximide to prevent further protein synthesis. Cells were treated with 2 ng/ml TGF- $\beta$ 1 for 0, 0.5, 1, 2, 3, and 7 hours. At the final time point, cells were washed in PBS, and 500  $\mu$ l 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  $-80\,^{\circ}$ C. The pellet was resuspended in 100  $\mu$ l of nuclear extraction buffer and rotated end-on-end at  $4\,^{\circ}$ 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- $\beta$ 1 (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- $\beta$ 1. RNA was reverse transcribed



using the Superscript III First-Strand Synthesis Kit (Invitrogen). The primers used to identify Smad7 were as follows: 5'-TGGCATACTGGGAGGAGAGAGAC-3'; 5'-CAGCCCTTCACAAAGCTGAT-3'. The primers produced a 400-bp product. DNA was amplified in a 25  $\mu$ l 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- $\beta$ 1. 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<sup>-/-</sup> 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'-GCT-GCGGGGAGAAGGGCGAC-3') of the human Smad7 complementary DNA sequence was synthesized in the sense and antisense orientation. For scratch-wound assays, 90% confluent *Itga3*<sup>-/-</sup> 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.

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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.  $P \le 0.05$  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.

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