nature medicine

Accelerated re-epithelialization in β_3-integrin-deficient mice is associated with enhanced TGF-β1 signaling

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The upregulation of TGF-β1 and integrin expression during wound healing has implicated these molecules in this process, but their precise regulation and roles remain unclear. Here we report that, notably, mice lacking β_3-integrins show enhanced wound healing with re-epithelialization complete several days earlier than in wild-type mice. We show that this effect is the result of an increase in TGF-β1 and enhanced dermal fibroblast infiltration into wounds of β_3-null mice. Specifically, β_3-integrin deficiency is associated with elevated TGF-β receptor I and receptor II expression, reduced Smad3 levels, sustained Smad2 and Smad4 nuclear localization and enhanced TGF-β1-mediated dermal fibroblast migration. These data indicate that $\alpha_v\beta_3$–integrin can suppress TGF-β1-mediated signaling, thereby controlling the rate of wound healing, and highlight a new mechanism for TGF-β1 regulation by β_3-integrins.

Wound healing involves coordinated infiltration of dermal cell types, together with extracellular matrix deposition and re-epithelialization[1]. It is influenced by a combination of growth factors, including transforming growth factor β1 (TGF-β1) and cell adhesion molecules such as integrins.

TGF-β1 controls cell behavior in a variety of cellular responses including cell proliferation, migration and extracellular matrix deposition, in addition to being a key coordinator of wound healing[1–6]. Loss of TGF-β regulation is associated with many human diseases including cancer progression and fibrotic disease[2,3,6]. Indeed, anti-TGF-β strategies are presently in clinical trials for treatment of fibrosis[7,8] and cancer, and exogenous TGF-β3 administration for wound healing treatment[9].

TGF-β1 acts through its receptors TGF-β RI and RII, followed by activation and nuclear translocation of its downstream effector molecules including Smad2, Smad3 and Smad4 (ref. 3), which in turn control transcription and cell behavior. The upregulation of TGF-β1 during wound healing has suggested that it regulates cutaneous repair[10], but the exact role for TGF-β1 in wound healing still remains unclear. TGF-β1 release from activated platelets at the wound site is thought to attract and activate macrophages and fibroblasts[11] whose infiltration is integrin dependent. These cells in turn secrete more TGF-β1, activating re-epithelialization and upregulation of further integrins[12,13]. Paradoxically, exogenous TGF-β1 inhibits keratinocyte proliferation *in vitro* and *in vivo*[5,10,14], an activity that can reduce wound healing rates *in vivo*[15,16] but enhance keratinocyte migration *in vitro*[5]. To complicate the matter further, application of TGF-β1 to wounds can either inhibit or enhance re-epithelialization, depending on the repair model and dose of cytokine used[17]. Moreover, genetic ablation of TGF-β1 or its receptors has been reported to either reduce[18] or enhance[10,19] the rate of wound repair. Understanding the regulation of TGF-β1 and its role in wound healing therefore is necessary to determine better therapeutic strategies.

Several members of the α_v integrin family are also thought to be involved in wound healing. Expression of $\alpha_v\beta_5$ and $\alpha_v\beta_6$ is upregulated in the epidermis during wound closure[13,20]. Expression of $\alpha_v\beta_3$ (a receptor for vitronectin and other extracellular matrix molecules) is elevated on several cell types that are involved in wound healing, including platelets, neovascularizing endothelial cells, macrophages and dermal fibroblasts[21,22]. Furthermore, inhibition of α_v integrin function using antagonists or inhibitory antibodies can prevent the migration of these cells in *in vitro* assays[23,24]. Moreover, administration of $\alpha_v\beta_3$ inhibitors[25] reduces granulation tissue formation and wound-induced angiogenesis, suggesting that $\alpha_v\beta_3$ is required for proper wound healing. In contrast to these studies, mice lacking β_5 or β_6 do not exhibit altered wound-healing rates[26,27], suggesting that the roles of α_v–integrin in wound healing is likely to be more complex than initially envisaged, and raise the question of the importance of $\alpha_v\beta_3$ in wound healing.

RESULTS

Re-epithelialization is enhanced in β_3-null mice

Wound healing and tumor growth share many common features. Because we reported previously that tumor growth is enhanced in β_3-integrin-deficient mice we examined wound healing in these

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Published online 16 January 2005; doi:10/nm1165

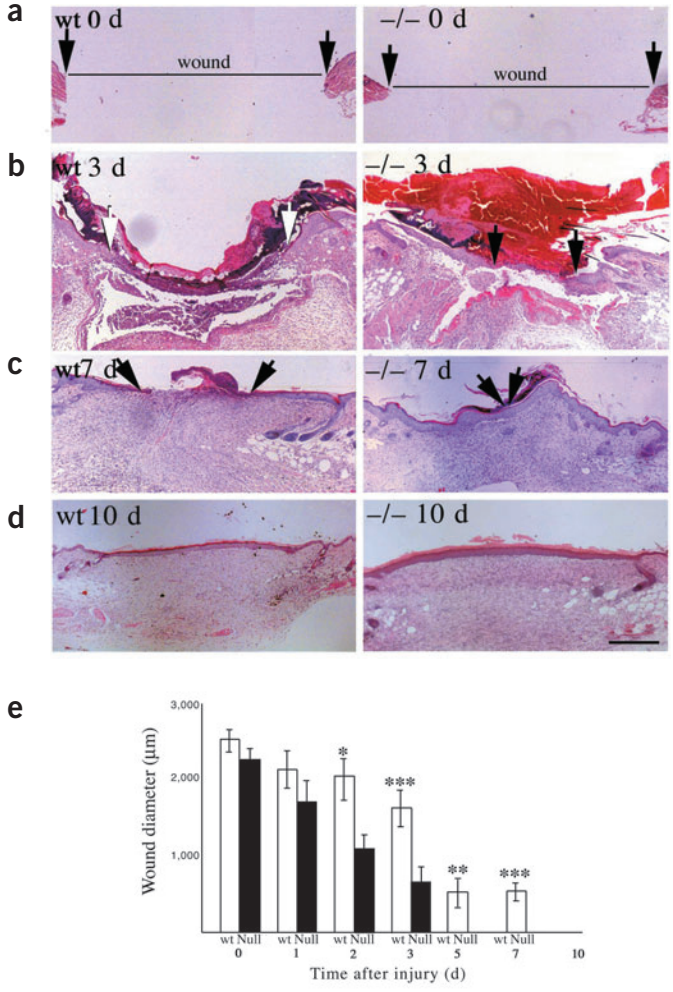

Figure 1 Re-epithelialization is accelerated in β_3-integrin–deficient mice. Sections of wounded skin from wild-type (wt) (**a**–**d**, left panels) and β_3-null (**a**–**d**, right panels) mice were stained with hematoxylin and eosin to examine re-epithelialization at day 0 (**a**), day 3 (**b**), day 7 (**c**) and day 10 (**d**) after injury. (**e**) Quantification of wound widths over 10 d after injury. Results represent the mean ± s.e.m; $n = 13$–14 for each time point and genotype; $^*P < 0.03$, $^{**}P < 0.04$, $^{***}P < 0.05$. Arrows indicate the leading edges of wounded epidermis. Open bars, wild-type; solid bars, β_3-null. Scale bar in **d**, 530 µm.

mice[28]. Furthermore, given that the level of β_3-integrin expression is elevated in platelets, endothelial cells, macrophages and dermal fibroblasts during wound healing, and that inhibitors of $\alpha_v\beta_3$ can suppress granulation tissue formation[25], β_3-integrin deficiency may affect adult cutaneous wound healing. Wild-type and β_3-integrin–deficient mice were given full-thickness wounds and subsequently harvested samples of wounded skin over 10 d. Sections of wounded skin were analyzed for wound closure (**Fig. 1a–d**). As shown in **Fig. 1**, wounds closed in β_3-integrin-deficient mice perfectly adequately, and re-epithelialization was accelerated significantly 2–7 d after wounding when compared with wild-type controls ($P < 0.03$–0.05, **Fig. 1e** and **Supplementary Fig. 1** online). The enhanced re-epithelialization in β_3-null mice was first observed at 2 d after wounding and by day 5, all the wounds in the mutant mice had closed (**Fig. 1e**). In contrast, complete re-epithelialization of wounds in wild-type mice was not observed until 10 d after injury (**Fig. 1d**). At this early stage of wound repair we did not observe any differences in granulation tissue area (**Supplementary Fig. 2** online) or fibroblast differentiation to myofibroblasts (**Supplementary Fig. 2** online) between wild-type and β_3-null wounds. Examination of myofibroblast numbers in immunostained sections showed very few myofibroblasts present in the granulation tissue at 3 d (data not shown), suggesting that wound contraction was not affected by β_3-integrin deficiency. Taken together, these data show that β_3-integrin is not essential for wound healing and moreover that deficiency in β_3-integrin enhances re-epithelialization.

Elevated TGF-β1 is associated with enhanced re-epithelialization

Because TGF-β1 is known to be involved in re-epithelialization and re-dermalization, and is produced in large amounts during wound healing, we wished to test whether the enhanced re-epithelialization in the β_3-null mice was associated with changes in TGF-β1 production. *In situ* hybridization for *Tgfb1* mRNA, which encodes TGF-β1, showed that its expression within the granulation tissue was enhanced in day 7 β_3-null wounds when compared with wild-type wounds (**Fig. 2a** and **Supplementary Fig. 3** online). The distribution of *Tgfb1* mRNA in these assays suggests that dermally derived cells, possibly including dermal fibroblasts, are involved in the elevated *Tgfb1* mRNA levels in the β_3-null wounds. We did not observe any *Tgfb1* mRNA in the skin of nonwounded mice from either genotype (data not shown). Concordant with these results, both *Tgfb1* mRNA and TGF-β1 protein levels were significantly higher in day 3 β_3-null wounded skin samples compared with wild-type controls ($P < 0.03$, **Fig. 2b**). Immunohistochemical analysis showed elevated TGF-β1 in the granulation tissue of β_3-null wounds (**Fig. 2c**). Notably, we could barely detect *Tgfb1* mRNA in nontreated skin from either wild-type or β_3-null mice, suggesting that the enhanced expression of TGF-β1 in β_3-integrin–deficient mice was injury induced. One indicator of elevated TGF-β1-mediated signaling is enhanced phosphorylation of downstream effectors such as Smad2. Immunohistochemical detection of phosphorylated Smad2 (pSmad2) showed increased expression in the granulation tissue of β_3-integrin–null wounds, and suggested that TGF-β1-mediated signaling was enhanced in β_3-null wounds (**Fig. 2c** and **Supplementary Fig. 4** online). To determine the cell types that showed elevated TGF-β1, we performed ELISAs on platelets, fibroblasts and macrophages isolated from nonwounded wild-type and β_3-null mice. β_3-null platelets released twofold more TGF-β1 than wild-type platelets with no difference in the levels of TGF-β1 observed in fibroblasts or macrophage releasates (**Fig. 2d**). We have shown previously that, despite their extended bleeding time, the numbers of circulating platelets in β_3-null mice are normal[29]. Thus, although not tested, we have no reason to believe that the platelet numbers in the wound *per se* differ between control and mutant mice. Because identical numbers of platelets were isolated from nonwounded control and mutant mice and used for the TGF-β1 ELISA, our results imply that β_3-integrin deficiency is associated with enhanced TGF-β1 release. We speculate that at the time of wounding, platelets are activated and TGF-β1 released at higher levels from the β_3-null platelets, which in turn initiates a positive feedback loop to accelerate the repair process.

Neutralization of TGF-β1 reduces wound closure

To confirm a specific role for TGF-β1 in accelerated re-epithelialization, we injected β_3-null wounds with a neutralizing antibody specific for TGF-β1 and harvested wounded skin 3 d after injury. Addition of the neutralizing antibody resulted in a decrease in the rate of re-epithelialization in β_3-null wounds compared with untreated control day 3 wounds

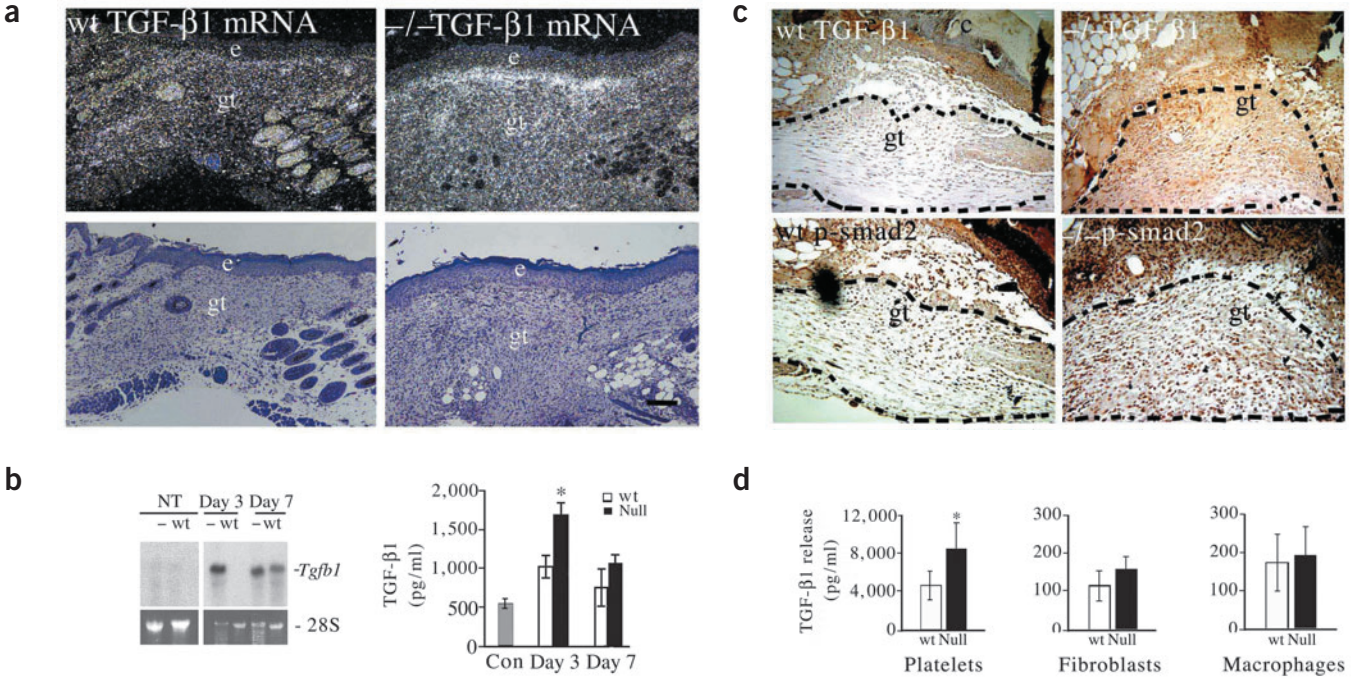

Figure 2 *Tgfb1* mRNA and TGF-β1 protein levels are elevated in β_3-integrin–deficient wounds. (**a**) *In situ* hybridization for *Tgfb1* mRNA was carried out on day 7 wild-type and β_3-null wound sections. Top panels show dark field images, bottom panels show corresponding bright field images. (**b**) Left, northern blot analysis of *Tgfb1* using mRNA extracted from nontreated (NT), day 3 and day 7 wounded skin samples from wild-type (wt) and β_3-null (–) mice. Right, day 3 and day 7 wounded wild-type and β_3-null skins were examined for amount of TGF-β1 by ELISA. Con, nonwounded control skin. Results represent the mean ± s.e.m.; $n = 9$–10 mice for each time point and genotype, $^*P < 0.03$. (**c**) Detection of TGF-β1 (top panels) and pSmad2 (bottom panels) in day 3 wounds of wt and β_3-null mice. Dotted lines encompass granulation tissue. e, epidermis; gt, granulation tissue; c, clot. Scale bar in **a**, 250 μm; in **c**, 50 μm. (**d**) TGF-β1 releasate was measured from platelets, macrophages and fibroblasts from nonwounded wild-type and β_3-null mice. $^*P < 0.03$.

(**Fig. 3a**,**b**). Quantification of the wound width confirmed that the TGF-β1 neutralizing antibody significantly reduced wound closure ($P < 0.005$, **Fig. 3c**). This result suggests strongly that the increased TGF-β1 observed in β_3-null wounds is responsible for accelerated wound healing in these mice.

Keratinocyte proliferation is decreased in β_3-null wounds

Reduced keratinocyte proliferation and increased extracellular matrix production are characteristic features of TGF-β1 activity during wound healing[5,16,17,30,31]. To test whether the elevated TGF-β1 levels in β_3-null wounds correlated with these features we analyzed proliferation by counting the numbers of Ki67- and 5-bromodeoxyuridine (BrdU)-positive keratinocytes in the hyperproliferative epidermal regions of β_3-null and wild-type wounds 3 d after injury (**Supplementary Fig. 5** online). The percentage of proliferating keratinocytes was significantly lower in β_3-null samples in both Ki67-stained and BrdU-labeled sections ($P < 0.03$, **Supplementary Fig. 5** online). In addition, we did not observe any difference in the level of apoptosis between the two genotypes (data not shown).

Before re-epithelialization can occur, a new dermis must first be made. Re-dermalization involves the deposition of new extracellular matrix (ECM) that serves as a substrate for infiltrating cell migration. We analyzed the deposition of fibronectin and vitronectin within the wound bed by immunofluorescence of day 3 wounded skin from both wild-type and β_3-null mice. Fibronectin and vitronectin deposition was greater in the β_3-null granulation tissue (**Supplementary Fig. 5** online). The deposition of other ECM molecules, including collagen type I, was normal in β_3-null wounds (data not shown). Thus, the accelerated re-epithelialization in β_3-null wounds correlates with reduced keratinocyte proliferation and enhanced fibronectin deposition, which are characteristic of TGF-β1-mediated responses.

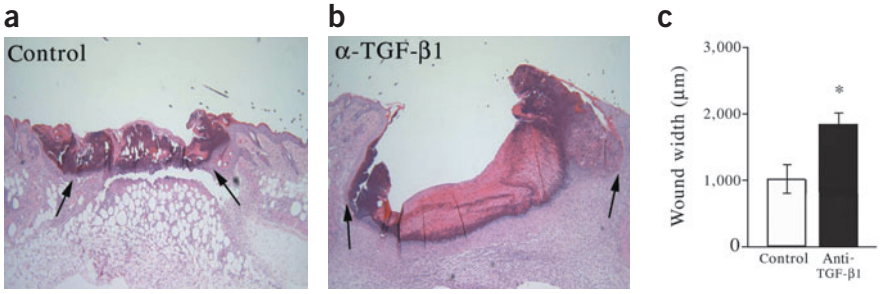

Figure 3 Re-epithelialization is decreased in β_3-null wounds injected with a TGF-β1 neutralizing antibody. β_3-null mice were injected with either PBS (**a**) or 50 μg/ml TGF-β1 neutralizing antibody (α-TGF-β1) (**b**) immediately before wounding and were killed 3 d after wounding. (**c**) Quantification of wound widths 3 d after injury with or without TGF-β1 neutralizing antibody. Results represent the mean ± s.e.m; $n = 4$–8 for each treatment; $^*P < 0.005$. Arrows indicate the leading edges of wounded epidermis. Magnification, ×50.

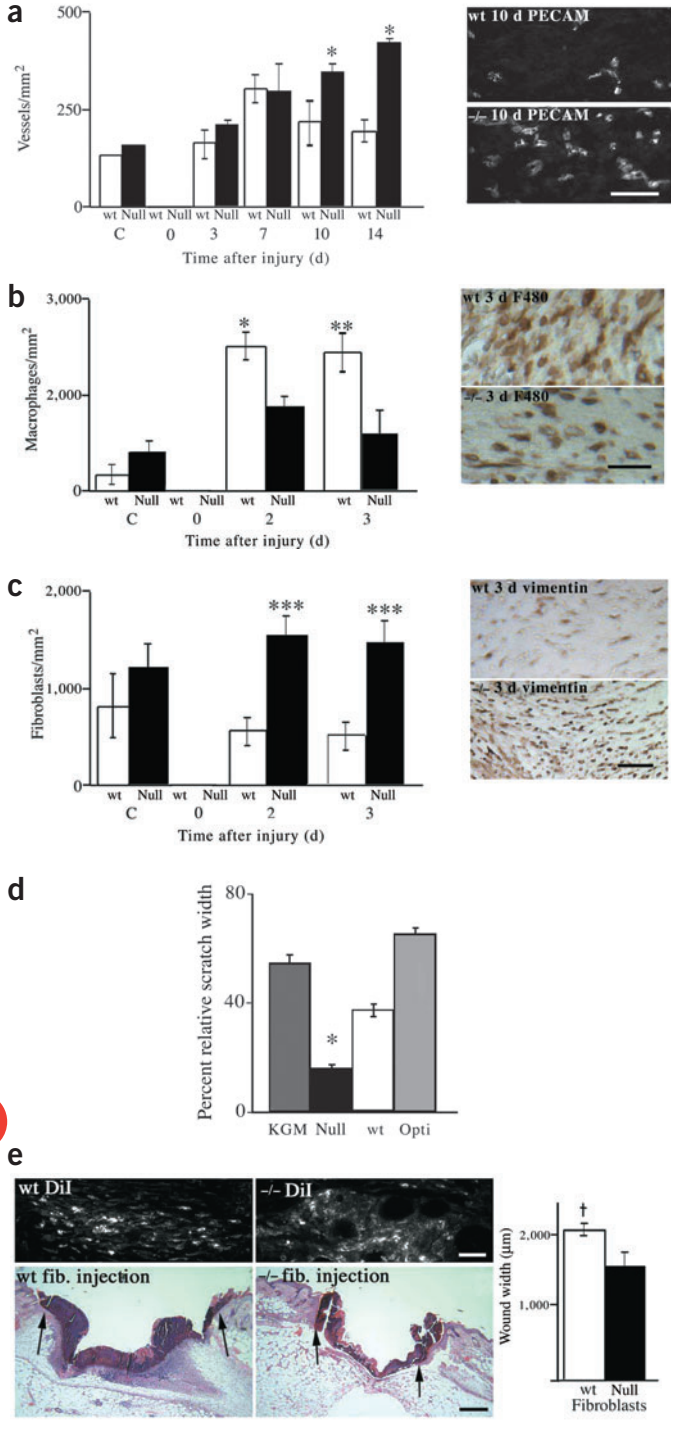

Figure 4 Dermal fibroblast infiltration is significantly elevated in β_3-null wounds and accelerates re-epithelialization. Numbers of blood vessels (**a**), macrophages (**b**) and dermal fibroblasts (**c**) were counted in untreated (C) skin or in the wound bed of wild-type and β_3-null mice various days after injury. (**d**) Scratch wounds were generated in wild-type keratinocyte cultures and were treated with either full keratinocyte growth medium (KGM), β_3-null fibroblast conditioned medium (null), wild-type fibroblast conditioned medium (wt) or Optimem (Opti). Results shown are mean ± s.e.m.; $n = 3$ for each time point and condition, $^*P < 0.005$. (**e**) DiI-labeled wild-type or β_3-null dermal fibroblasts were detected in paraffin sections (top panels) within the wound bed of wild-type animals 3 d after administration. Representative hematoxylin and eosin–stained sections of wounds containing wild-type or β_3-null dermal fibroblasts are shown (bottom panels). Results are mean ± s.e.m.; $n = 4$–6 for each time point and genotype; $^{***}P < 0.005$, $^{**}P < 0.03$, $^*P < 0.01$ $\dagger P < 0.06$. Open bars, wild type; solid bars, β_3-null. Scale bar in **a**, 50 μm; in **b**, 40 μm; in **c**, 60 μm; in **e**, 50 μm (top) and 250 μm (bottom).

Enhanced wound healing is associated with elevated dermal fibroblast infiltration

Because cutaneous wound healing can be attributed to several cell types, we examined which cell types were involved in the accelerated repair response in β_3-null mice. Keratinocytes do not express β_3-integrin *in vivo* or *in vitro*[12] (and data not shown), therefore we reasoned that molecular compensation within the epidermis was unlikely to be the cause of accelerated re-epithelialization and that enhanced re-dermalization was more likely to be responsible. Thus we examined dermal cell types that express β_3-integrin, namely, endothelial cells in angiogenic vessels, macrophages and dermal fibroblasts.

Angiogenesis is necessary in wound healing to sustain the newly formed granulation tissue and we have shown previously that tumor angiogenesis is enhanced in β_3-integrin–deficient mice[28]. We examined angiogenesis, using PECAM to identify endothelial cells, in the β_3-null and wild-type wounds. In control skin, and up to 7 d after injury, we did not observe any significant difference in the number of blood vessels between wild-type and β_3-null samples (**Fig. 4a**). After this time, the number of blood vessels was higher in β_3-null wounds. But the elevated neovascularization occurred much later than enhanced re-epithelialization and therefore is probably not the primary cause for accelerated wound healing in these mice.

Macrophage and neutrophil infiltration into the wound site is thought to be important for debridement and provides a source of growth factors to repairing skin. Although no differences in macrophage numbers were observed between nonwounded control wild-type and β_3-null skin, the number of macrophages in β_3-null wounds was significantly lower than in wild-type wounds ($P < 0.005$, **Fig. 4b**). Neutrophil numbers remained unchanged in both wild-type and β_3-null day 3 wounds (**Supplementary Fig. 6** online). The results suggest that β_3-integrin deficiency inhibits macrophage homing and/or residence during wound healing with no difference in baseline levels, but neutrophil infiltration is not affected.

Lastly, because fibroblasts are the main source of ECM deposition and reorganization within the wound bed, we examined dermal fibroblast infiltration into the granulation tissue. We observed that although fibroblast numbers in untreated, control skin were similar between β_3-null and wild-type dermis, β_3-null dermal fibroblast recruitment in wound tissue was significantly elevated when compared with wild-type controls ($P < 0.005$, **Fig. 4c**). Hence, enhanced recruitment of β_3-null dermal fibroblasts to the wound bed may be responsible for enhanced wound healing in the β_3-null mice.

To examine whether the β_3-null dermal fibroblasts secrete factors involved in enhanced re-epithelialization, we scratched cultured keratinocytes and treated them with conditioned medium from wild-type or β_3-null dermal fibroblasts. Wounded keratinocytes exposed to β_3-null conditioned medium closed significantly faster than keratinocytes grown in wild-type conditioned medium ($P < 0.005$, **Fig. 4d**).

We then injected dioctadecyltetramethylindocarbocyamine perchlorate–labeled wild-type or β_3-null dermal fibroblasts into the wound site of wild-type mice and examined re-epithelialization 3 d later (**Fig. 4e**). The administration of β_3-null fibroblasts was sufficient to accelerate re-epithelialization, implying that β3 integrin–deficient fibroblasts enhance wound healing by secreting soluble factors, including TGF-β1, that accelerate wound healing by stimulating keratinocyte migration.

β_3-integrin deficiency enhances TGF-β1 signaling

Because TGF-β1 levels and dermal fibroblast infiltration were elevated in β_3-null wounds, we examined the total protein levels of TGF-β RI, TGF-β RII and downstream Smad signaling in wild-type and β_3-null dermal fibroblasts. Although we detected no differences in total levels of Smad2 (**Fig. 5a**) and Smad4 (**Fig. 5b**), pSmad2 was significantly increased in β_3-null fibroblasts ($P < 0.05$, **Fig. 5c**) and Smad3 levels were significantly reduced ($P < 0.02$, **Fig. 5d**). Moreover, a twofold increase in total TGF-β RI and TGF-β RII levels was observed in the β_3-null fibroblasts (**Fig. 5e**,**f**). These results indicated that β_3-integrin could be important in the regulation of TGF-β1-mediated signaling molecules. Given that the extent of Smad nuclear translocation is directly proportional to TGF-β receptor activity[32], we examined nuclear translocation of Smad2, Smad3 and Smad4 in the β_3-null and wild-type fibroblasts. Nuclear translocation of Smad2 and Smad4 peaked at 0.5–1 h after TGF-β1 treatment in wild-type fibroblasts and then regressed. In β_3-null fibroblasts, nuclear translocation of these Smads was sustained at significantly higher levels than observed in wild-type controls ($P < 0.008$, **Fig. 6a**,**b**). In contrast, Smad3 nuclear translocation was significantly reduced in β_3-null fibroblasts when compared with wild-type controls ($P < 0.008$, **Fig. 6c**). The expression of Smad2, Smad3 and Smad4 in cytoplasmic extracts of these cells showed inverse responses to the nuclear profiles (data not shown). Taken together, these results suggest that β_3-null dermal fibroblasts may be more responsive to TGF-β1. This was confirmed when, in the presence of TGF-β1, β_3-null fibroblasts significantly enhanced migration ($P < 0.03$) when compared with wild-type dermal fibroblasts (**Fig. 6d**). We then asked whether the apparent increase in migration was the result of elevated proliferation and/or compensatory integrin expression and function in β_3-null fibroblasts. We detected no differences in fibroblast proliferation or surface integrin expression and function of non-β_3-integrins when comparing wild-type with β_3-null fibroblasts *in vitro*[29] (and data not shown). Notably, migration of wild-type and β_3-null dermal fibroblasts in the absence of TGF-β1 showed no significant difference (**Fig. 6d**), suggesting that any increase in TGF-β1-mediated migration was probably the result of elevated TGF-β1-mediated responses *per se*.

Our data provide strong evidence for a new role for β_3-integrin in the control of TGF-β1-mediated responses by affecting TGF-β RI, TGF-β RII and downstream Smad signaling events that are likely to be contributing to the enhanced wound-healing response in the β_3-deficient mice.

DISCUSSION

Considering the importance of $\alpha_v\beta_3$–integrin in cell migration and its interactions with its ligands, including vitronectin, fibronectin and osteopontin, all of which are upregulated during cutaneous repair[20,21], the accelerated re-epithelialization observed in β_3-null mice is surprising. β_3-null mice are the first integrin-deficient mouse model reported to have such a phenotype[26,27,33]. In addition, in the osteopontin and vitronectin doubly deficient mice, no changes in re-epithelialization were observed[34]. Our results therefore suggest a specific and independent role for β_3-integrin in wound healing.

TGF-β1 levels are upregulated significantly during wound healing, is a mitogen for fibroblasts and endothelial cells and is thought to enhance wound healing through these effects[35]. But studies involving transgenic and knockout mouse models have yet to define the precise role of TGF-β1 in wound healing[10,19,36]. Here we report that elevated levels of TGF-β1 are associated with accelerated re-epithelialization in β_3-null mice. Examination of TGF-β1 released from wild-type and β_3-null platelets, isolated from nonwounded mice, revealed a twofold increase in the amount of TGF-β1 released from β_3-null platelets. One possible explanation for this observation is that β_3-integrin deficiency in platelets disrupts the normal PI3-kinase signaling pathways which have been shown to be involved in TGF-β1 release[37]. We have evidence that this signaling pathway is disrupted in β_3-null endothelial cells[38] and thus examination of PI3-kinase pathways may provide some insight into the regulation of TGF-β1 release from β_3-null platelets. This increased level of TGF-β1 was evident at the time when re-epithelialization was accelerated in β_3-null wounds. These data suggest that increased TGF-β1 release from platelets leads to increased TGF-β1 protein in wounds and enhanced re-epithelialization. In addition, it has been shown that TGF-β1 can accelerate keratinocyte migration without an increase in proliferation[5], and these data correlate with the decreased level of keratinocyte proliferation observed in β_3-null mice. Moreover, neutralization of TGF-β1 with a specific TGF-β1-blocking antibody significantly reduced the rate of re-epithelialization in β_3-null wounds. Thus, our data suggest strongly that, at least in β_3-null mice, increased TGF-β1 expression enhances re-epithelialization.

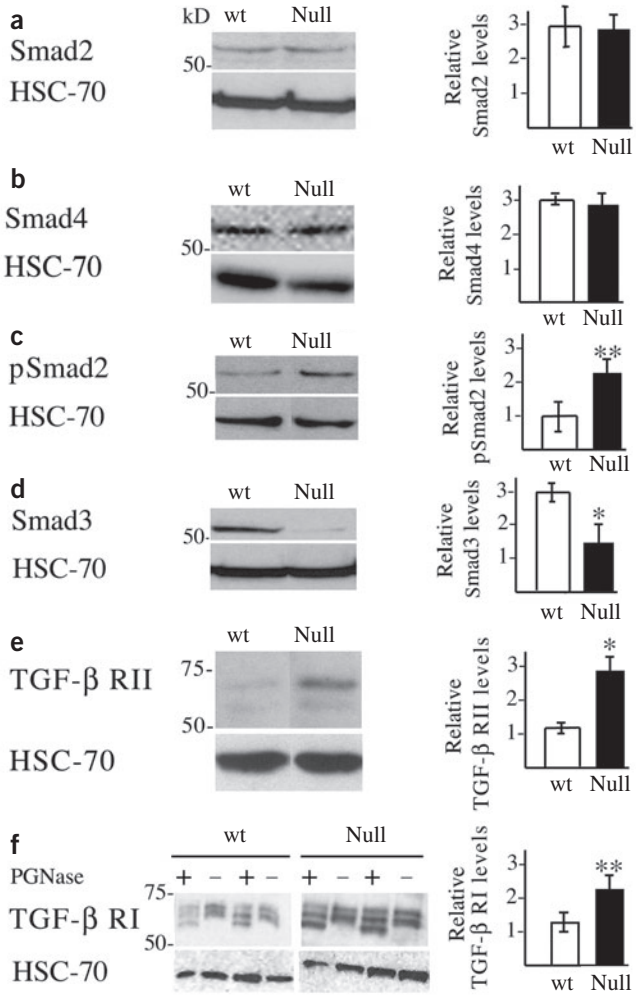

Figure 5 β_3-deficient dermal fibroblasts have elevated levels of TGF-β RI, TGF-β RII, increased pSmad2 and decreased levels of Smad3. Western blot analysis of protein extracts from wild-type (wt) and β3-null (Null) dermal fibroblasts for Smad2 (**a**), Smad4 (**b**), pSmad2 (**c**), Smad3 (**d**), TGF-β RII (**e**) and TGF-β RI (**f**). Duplicate lysates for TGF-β RI were either treated (+) or not treated (–) with PGNase. Bar charts represent densitometry results, means ± s.e.m., $n = 3$–4 independent experiments. HSC-70 provided loading controls. $^*P < 0.02$, $^{**}P < 0.05$. Open bars, wild-type; solid bars, β_3-null.

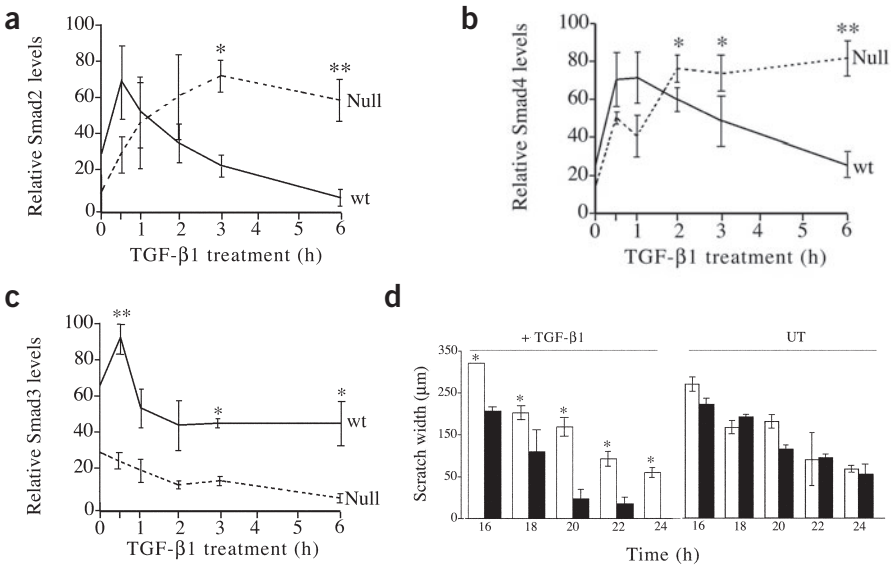

Figure 6 β_3-integrin deficiency enhances nuclear translocation of Smad2 and Smad4 and accelerates fibroblast migration in the presence of TGF-β1. Fibroblasts were treated with TGF-β1 (2 μg/ml) and nuclear extracts were analyzed by western blotting for Smad2 (**a**), Smad4 (**b**) and Smad3 (**c**). Graphs represent densitometry results relative to PCNA loading controls (mean) from three independent experiments. Wild-type and β_3-null dermal fibroblast cultures, in the absence or presence of TGF-β1 (1 ng/ml) were examined at various time points after scratch wounding, and wound width was measured (**d**). Results shown are mean ± s.e.m.; $n = 3$ for each time point and genotype; *P ranges from 0.008 to 0.05. Hatched line, β_3-null; solid line, wild-type; UT, untreated.

Because $\alpha_v\beta_3$ is not expressed by wild-type keratinocytes, the accelerated re-epithelialization in β_3-null mice was probably a consequence of enhanced re-dermalization, involving three of the major cell types thought to be involved in re-dermalization: endothelial cells in angiogenesis, macrophages and dermal fibroblasts.

Antagonists to β_3-integrin can either inhibit angiogenesis in the early stages of wound healing and prevent proper granulation tissue formation[25,39] or have no significant effect on wound healing (personal communication, S. Goodman). Our present data show enhanced angiogenesis in β_3-null wounds and corroborate our previous findings. However, the enhanced angiogenesis is probably not the cause for enhanced wound healing, because it occurs several days after the accelerated re-epithelialization event was detected.

We analyzed macrophage numbers in the wound bed since macrophages are thought to be responsible for the production of several growth factors, especially TGF-β1 (refs. 11,40). We observed a reduction in the number of macrophages in the β_3-null wound bed. This may be the result of inhibited migration simply because of the absence of β_3-integrin. Notably, it has been shown recently that macrophages may not be required for wound healing[41] and therefore their precise role in wound healing is unclear. We therefore conclude that changes in the number of macrophages in the wound bed, at least in β_3-null mice, do not affect re-epithelialization.

Dermal fibroblasts are important in the production of growth factors and ECM deposition during wound healing. We observed that in β_3-null mice, elevated dermal fibroblast numbers were detected 2–3 d after injury, correlating with enhanced ECM deposition and the time of accelerated re-epithelialization. Therefore the elevated fibroblast numbers may be responsible for the 'enriched' neodermis, aiding the accelerated re-epithelialization in the β_3-null wounds. Indeed, injection of β_3-null dermal fibroblasts into wounds in wild-type mice accelerated re-epithelialization, and β_3-null fibroblast conditioned medium increased keratinocyte migration *in vitro*, suggesting strongly that β_3-null dermal fibroblasts function to enhance wound healing. This led us to ask, what is the mechanism for increased dermal fibroblast infiltration in the β_3-null wounds?

Dermal fibroblasts may originate from other sources, including the bone marrow[42]. It is therefore conceivable that bone marrow mobilization may be enhanced in the β_3-null mice; however, such a possibility would not explain the enhanced TGF-β1-mediated migration observed *in vitro*. Alternatively, the absence of β_3-integrin could protect fibroblasts from apoptosis[43]. But TUNEL detection of apoptosis in the dermis showed no difference between wild-type and β_3-null wounds (data not shown) and this implies that β_3-integrin deficiency does not affect apoptosis, at least within the dermis.

Although there is no upregulation in the levels or activity of other fibroblast integrins[29] (and data not shown), compensation by other molecules prevails. Our data show that β_3-integrin deficiency is associated with enhanced TGF-β1-mediated fibroblast migration. These cells expressed elevated TGF-β RI and TGF-β RII, together with changes in downstream signaling molecules, (*i.e.*, Smads). These included decreased Smad3 levels and sustained nuclear localization of Smad2 and Smad4. Previous reports have shown that extended nuclear localization of Smad2 and Smad4 is directly proportional to the stabilization of the receptor at the cell surface and that this corresponds with elevated TGF-β1-mediated responses[32]. Hence the increased Smad nuclear localization and enhanced TGF-β RI and RII levels in β_3-null fibroblasts are likely to be the mechanisms responsible for their elevated TGF-β1-mediated migration and perhaps for their enhanced recruitment into the β_3-null wound bed.

Considering the many parallel roles of Smad2 and Smad3, it is notable that Smad3 levels are reduced in the β_3-null cells. Recently, however, differences in Smad2 and Smad3 functions and activity have been highlighted[44] and elevated TGF-β1-mediated responses have been shown to downregulate Smad3 expression[45]. In addition, accelerated re-epithelialization has been reported for Smad3-deficient mice, whereas Smad2 heterozygous mice have inhibited re-epithelialization[36]. Thus, the accelerated wound healing in β_3-null mice correlates well with a decrease in Smad3 levels, an increase in Smad2 and Smad4 nuclear localization and the elevated pSmad2 observed in the wound bed. Although studies have implicated TGF-β1 in altering integrin expression levels[12,13,46] and $\alpha_v\beta_6$–integrin (an epidermal-specific integrin) can activate the latent form of TGF-β1 (ref. 47), little is known about integrin regulation of TGF-β receptors and downstream signaling. Our results suggest a novel mechanism of TGF-β1-signaling regulation by β_3-integrin. One hypothesis is that $\alpha_v\beta_3$ expression can act as a transdominant inhibitor of TGF-β RI and RII and downstream signaling. This mechanism may normally function to regulate these two pathways under physiological conditions. Transdominant inhibition of β_3-integrin on other molecules such as $\alpha_5\beta_1$–integrin, a fibronectin receptor[48], and other growth factor receptors such as the vascular endothelial growth factor receptor have been previously reported[28].

It is noteworthy that other integrins have also been associated with regulation of TGF-β1 signaling. For example, overexpression of the laminin receptor $\alpha_6\beta_4$–integrin in suprabasal keratinocytes is associated with elevated tumor growth and reduced Smad2 phosphorylation[49].

Although we cannot rule out other possible mechanisms, we propose that the enhanced re-epithelialization observed in β_3-null mice is likely to be caused by an increased TGF-β1-mediated response by β_3-null dermal fibroblasts, resulting in enhanced fibroblast infiltration in combination with elevated ECM deposition and TGF-β1 expression. Our data also provide a better understanding of the regulation of TGF-β1-mediated signaling by β_3-integrin during wound healing. Considering the use of TGF-β1 in clinical trials in the treatment of wound healing, fibrosis and cancer, our data shed light on possible new therapeutic strategies for TGF-β1 regulation by integrins.

METHODS

For further details on methods, please see **Supplementary Methods** online.

Antibodies. Rabbit antiserum to fibronectin was provided by R. Hynes. Rabbit antisera to vitronectin was a gift from S. Curriden. Antibody specific for mouse PECAM was purchased from BD Pharmingen, and anti-macrophage antibody, F4-80, was obtained from Serotec. Anti-vimentin antibody was purchased from Sigma. Anti-Smad2/3 antibody was purchased from BD Transduction Labs, anti-Smad3 antibody and TGF-β RII were purchased from Upstate Biotechnologies and the antibody to pSmad2 was purchased from Cell Signalling Technologies. Antibodies to Smad4, HSC-70 and TGF-β RI were purchased from Autogen Bioclear. Proliferating cell nuclear antigen (PCNA) and Ki67 were obtained from Cancer Research UK. For immunohistochemical analysis, TGF-β1-specific antibody was a gift from A. Roberts and antibody to pSmad2 was from C-H. Heldin. The neutralizing TGF-β1 antibody was purchased from R&D Systems. Biotin-conjugated antibodies and all FITC- and horseradish peroxidase–conjugated secondary antibodies were purchased from Biosource International.

Wound healing experiments. One-month-old mice (β_3-null and wild-type, 129Sv pure background) were anesthetized and the dorsum shaved then cleaned with alcohol. Two 3-mm full-thickness cutaneous biopsy punch wounds were made either side of the midline of the mouse. Wounds were made maintaining the underlying fascia. The wounded tissue was collected on various days after injury. Tissue was bisected and either fixed in 10% formalin, or acid alcohol (96% ethanol/1% acetic acid) for paraffin embedding, or snap-frozen in OCT (Thermo Lifesciences).

***In vitro* wound-closure (scratch) assays.** Fibroblasts from wild-type and β_3-null newborn mice were isolated as described[50]. Plated fibroblasts were allowed to reach 70–80% confluence, followed by 24 h serum starvation. Scratches were made across the diameter of each well with a plastic pipette tip, and cells were either treated with 1 ng/ml TGF-β1 (Peprotech) or not. Scratch closure was measured using a grid inserted in the eyepiece of a Zeiss Telaval microscope (Zeiss).

For conditioned media experiments, wild-type keratinocytes were grown to confluence. Keratinocytes were serum starved in Optimem (Gibco) for 24 h. We removed the medium from log-phase growing, confluency-matched wild-type and β_3-null dermal fibroblasts and replaced it with Optimem for 24 h. Serum-starved keratinocytes were scratched as described above and the medium was replaced with either full keratinocyte growth medium (KGM), β_3-null fibroblast conditioned medium, wild-type fibroblast conditioned medium or Optimem. Scratch closure was measured as above.

TGF-β1 ELISA of wound tissue. We homogenized wounded skin in 2.5 N acetic acid containing 10 M urea, 1 mM PMSF and 10 μg/ml pepstatin A at room temperature for approximately 3 min. After a 20-min incubation at room temperature the samples were centrifuged at 13,000 rpm for 10 min and the supernatants were used in the TGF-β1 ELISA (according to manufacturer's instructions, R&D Systems).

TGF-β1 ELISA of isolated cells. Platelets were isolated from nonwounded wild-type and β_3-null mice as described previously[32]. An equal number of platelets from both genotypes, counted with a Neubauer hemocytometer, were analyzed for TGF-β1 release. For macrophage isolation and stimulation, femurs from wild-type and β_3-null mice were removed, cleaned and bone marrow flushed through with DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated fetal calf serum, and 10% conditioned supernatant from L929 cells, which was changed on day 2 and every 3 d subsequently. Macrophages were used between day 7 and 10 of culture. Macrophages were stimulated with lipopolysaccharide (0.5 μg / ml) (*Escherichia coli* serotype 026:B6, Sigma) and apoptotic human neutrophils (2.5×10^6 cells/well; corresponds to a 1:5 ratio of macrophages: apoptotic cells) and supernatants were harvested after 24 h.

We isolated fibroblasts as previously described from wild-type and β_3-null mice. Fibroblasts were grown to 70% confluence in DMEM + 10% fetal calf serum, followed by incubation in serum-free medium for 24 h. We removed the supernatant and centrifuged the samples at 2,000 rpm to remove cellular debris. We stored samples at –80 °C before analysis. Acid activation of the platelet releasate, fibroblast supernatant and macrophage releasate was required immediately prior to use of the TGF-β1 ELISA kit.

DiI labeling and fibroblast injection. Wild-type and β_3-null fibroblasts were grown to 70% confluence, and incubated with the cell tracker CM-DiI (Molecular Probes) for 5 min at 37 °C followed by an additional 15 min at 4 °C. After labeling, we washed the cells and resuspended them in PBS. We injected 5,000 wild-type or β_3-null cells intradermally at the edge of the wound site of wild-type mice 24 h after injury. Wounded skin was harvested 3 d after injury. Wounded tissue was fixed and stained with hematoxylin and eosin or dewaxed to identify DiI-labeled fibroblasts within the dermis.

TGF-β1 neutralizing antibody injection. Mice were wounded as previously described. Prior to wounding, we injected 50 μg/ml TGF-β1 neutralizing antibody intradermally into a premarked site on the shaved dorsum of β_3-null mice. Immediately after injection, a wound was created adjacent to the site of injection using a 3-mm biopsy punch. Although two wounds were generated per mouse, only the right-hand wound received the TGF-β1 neutralizing antibody. The left-hand wound was used as an internal control. Another group of β_3-null mice were injected with PBS, as a further control. Three days after wounding, we harvested the wounds and stained paraffin-embedded sections with hematoxylin and eosin. Wound diameter was measured as previously described.

Animals. All animals were used in accord with United Kingdom Home Office regulations.

Statistical analysis. All numerical results are presented as mean ± s.e.m. The significance between two data sets was tested with unpaired *t* tests. Differences were considered significant when $P < 0.05$.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGEMENTS

We thank C. Hill, G. Inman F. Parkinson, I. Hart, F. Watt, D. Owens and A. Daley for their helpful advice and criticisms throughout the work; S. Watling and C. Wren for their technical assistance; G. Elias and colleagues for help with histology; F. Balkwill's laboratory for technical assistance.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 30 March; accepted 1 December 2004
Published online at http://www.nature.com/naturemedicine/

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