

Wound Healing in the $\alpha 2\beta 1$ Integrin-Deficient Mouse: Altered Keratinocyte Biology and Dysregulated Matrix Metalloproteinase Expression

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The $\alpha 2\beta 1$ integrin, a collagen/laminin receptor, is expressed at high level in the basal cell layer of the epidermis. To define the role of the $\alpha 2\beta 1$ integrin in wound healing, wound repair was extensively evaluated in wild-type and $\alpha 2$ -null mice *in vivo*. In addition, the impact of $\alpha 2\beta 1$ integrin-deficiency on the function of primary murine keratinocytes *in vitro* was analyzed. Our *in vivo* findings demonstrate that genetic deletion of the $\alpha 2\beta 1$ integrin does not significantly alter the rate of re-epithelialization, collagen deposition, or tensile strength during wound closure in mice. In marked contrast to the observed similarities in wound healing, deletion of the $\alpha 2\beta 1$ integrin resulted in a dramatic increase in neoangiogenesis in the wound microenvironment. In contrast to *in vivo* studies, primary keratinocytes from $\alpha 2$ -null mice adhered poorly and displayed impaired migration on type I collagen *in vitro*. We demonstrate that $\alpha 2\beta 1$ integrin-ligation negatively regulates expression of genes including matrix metalloproteinases both *in vivo* and *in vitro*. Furthermore, the changes in gene expression could potentially account for relatively normal wound healing in the $\alpha 2$ -deficient mouse and our recent observation that suggests an antiangiogenic role for the $\alpha 2\beta 1$ integrin *in vivo*.

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INTRODUCTION

The $\alpha 2\beta 1$ integrin is the most abundantly expressed collagen receptor on resting keratinocytes, where it is highly expressed in the basal layer of the epidermis. The multiple members of the $\beta 1$ integrin family mediate maintenance of the epithelial barrier, adhesion of the epidermis to the basement membrane, epithelial migration across the dermal extracellular matrix during development and wound healing, and signals that maintain keratinocyte stem cells within the cell cycle for normal self-renewal and those that stimulate epithelial differentiation. Mice with keratinocyte-specific deletion of the $\beta 1$ integrin gene have a mild blistering disorder, partial hair loss with diminished hair follicles, impaired epidermal proliferation, and a mild defect in wound healing (Brakebusch

et al., 2000; Grose *et al.*, 2002). Keratinocytes from mice with targeted deletion of the $\beta 1$ integrin gene demonstrate altered expression of other genes including matrix metalloproteinase 10 (MMP-10; stromelysin-2), MMP-13 (collagenase-3), and the $\beta 4$ integrin subunit. Deletion of the $\beta 1$ subunit results in loss of expression of several α - β heterodimers; the contribution of individual α - β heterodimers to the changes observed in the $\beta 1$ -deficient mouse have not been defined.

In vitro studies of human skin and cell lines have suggested that the $\alpha 2\beta 1$ integrin is involved in wound healing (Saarialho-Kere *et al.*, 1993; Dumin *et al.*, 2001). During re-epithelialization of a wound, keratinocytes dissolve their hemidesmosomal attachments to the basal lamina. The $\alpha 2\beta 1$ integrin is relocalized from the basal cell area and concentrated at the forward leading edge of migrating keratinocytes bringing the integrin in contact with type I collagen (Martin, 1997). Keratinocyte migration on complex matrices containing type I collagen requires the catalytic activity of collagenase I (MMP-1). Expression of MMP-1 is upregulated in human basal keratinocytes by the interaction of the $\alpha 2\beta 1$ integrin with type I collagen in the underlying dermis. Keratinocyte migration on type I collagen is inhibited by anti-MMP-1 antibodies and by inhibitory antibodies against the $\alpha 2\beta 1$ integrin (Pilcher *et al.*, 1997).

We previously reported that wound healing occurred normally in the $\alpha 2\beta 1$ integrin-deficient mouse. In that earlier study, a 100 mm² full-thickness wound was placed on the flank of wild-type and $\alpha 2$ -null animals. Compared to

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Abbreviations: H&E, hematoxylin and eosin; MMP, matrix metalloproteinase; qRT-PCR, quantitative real-time PCR

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wild-type animals, $\alpha 2$ -deficient mice demonstrated no defect in re-epithelialization. Both wild-type and $\alpha 2$ -null animals displayed a similar temporal pattern of wound closure (Chen *et al.*, 2002). In fact, wound closure of these 100 mm² wounds was slightly more rapid in the absence of the $\alpha 2\beta 1$ integrin. This observation was rather surprising, as it appeared to contradict earlier *in vitro* studies using inhibitory antibodies.

To evaluate further the impact of $\alpha 2\beta 1$ integrin deletion on wound healing, the ability to repair wounded wild-type and $\alpha 2$ -null epidermis has now been examined more extensively using a number of different *in vivo* models and primary keratinocytes *in vitro*. Our *in vivo* findings demonstrate that the $\alpha 2\beta 1$ integrin does not play an essential role in re-epithelialization. However, our data suggest that the $\alpha 2\beta 1$ integrin plays a critical role in regulating neoangiogenesis within the wounded microenvironment. Utilizing primary keratinocytes from wild-type (+/+) and $\alpha 2$ -null (-/-) mice to dissect the consequences of $\alpha 2\beta 1$ integrin deficiency, we

now report studies describing the role of the integrin in keratinocyte adhesion and migration, as well as gene expression. Our *in vitro* findings reveal that loss of the $\alpha 2\beta 1$ integrin negatively regulates the expression of a number of genes including several MMPs.

RESULTS

Enhanced angiogenesis in the $\alpha 2\beta 1$ integrin-deficient mouse

In our initial report describing the phenotype of the $\alpha 2\beta 1$ integrin-deficient mouse we demonstrated that deficiency of the $\alpha 2\beta 1$ integrin failed to alter the rate of wound closure, specifically re-epithelialization of a 100 mm² excisional wound. To further evaluate the impact of $\alpha 2\beta 1$ integrin deletion on wound healing, the ability to repair wild-type and $\alpha 2$ -null epidermis was evaluated extensively using a number of wounding models including full-thickness 6 mm² excisional wounds, linear incisions, and subcutaneous sponge implants placed on the flank. As shown in Figure 1a, closure of full-thickness 6 mm² excisional wounds proceeded at

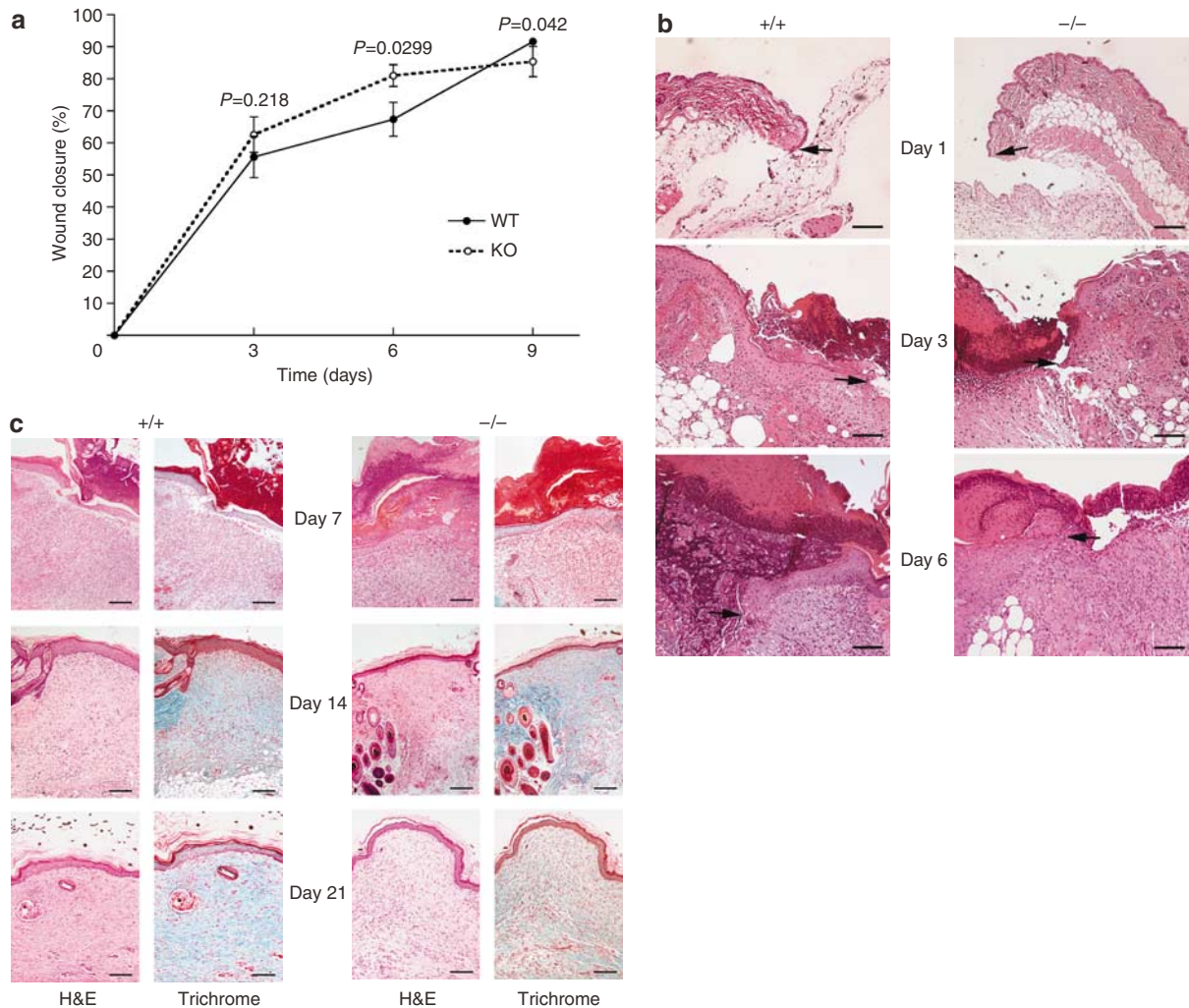


Figure 1. Wound closure in wild-type and $\alpha 2$ -null mice. (a) The rate of wound closure of full-thickness 6 mm² excisional wounds proceeded at a similar rate in both wild-type and $\alpha 2$ -null mice. (b) Morphologic evaluation of H&E-stained 6 mm² excisional wounds from wild-type (+/+) and $\alpha 2$ -null (-/-) mice on post-wound days 1, 3, and 6 (bar = 100 μ m) (original magnification $\times 200$). (c) Morphologic evaluation of H&E and Gomori's trichrome stained 6 mm² excisional wounds from wild-type (+/+) and $\alpha 2$ -null (-/-) mice on post-wound days 7, 14, and 21 (bar = 100 μ m) (original magnification $\times 200$).

a similar rate in both wild-type animals and animals lacking the $\alpha 2\beta 1$ integrin on a pure C57/BL6 background. However, at day 6 there was a slight but statistically significant ($P=0.029$) increase in the extent of wound closure in the $\alpha 2$ -null animals. These data comparing the closure of 6 mm² excisional wounds in wild-type and $\alpha 2$ -null animals on a pure C57/BL6 background confirm our previously reported observations of the rate of closure of a 100 mm² excisional wound in the mixed C57/BL6/SVJ120 animals. The slightly increased extent of re-epithelialization in the $\alpha 2$ -null animals at day 6 was also similar to our previously published data. In addition, these observations suggest that the background studied, either C57/BL6 or mixed SVJ129/C57/BL6, does not affect wound closure.

Morphologic analysis of wild-type and $\alpha 2\beta 1$ integrin-deficient skin wounded by 6 mm² excision was carried out at multiple time points. As shown in Figure 1b, there was no detectable morphologic difference between wild-type and $\alpha 2$ -null mice at 24 hours. Only mild dermal edema and scattered inflammatory cells in the dermis were detectable in both wild-type and $\alpha 2$ -null animals. At day 3 a large fibrin clot with a robust inflammatory response covered the wound area in both genotypes (Figure 1b). The extent and composition of the immune cell infiltrate, as assessed by morphologic evaluation of hematoxylin and eosin (H&E)-stained sections and immunohistochemical analysis with antibodies directed against CD45 or Gr-1 that recognize leukocytes or neutrophils, respectively, was similar between wild-type and $\alpha 2$ -null mice. By day 6, re-epithelialization was nearing completion and the dermal matrix was beginning to

reorganize. The granulation tissue at both days 3 and 6 was very cellular in animals of both genotypes. In contrast to these similarities the degree of vascularization differed between wild-type and $\alpha 2$ -null animals. There appeared to be increased vessel number and area in the $\alpha 2\beta 1$ integrin-null mouse.

Previous *in vitro* studies of the $\alpha 2\beta 1$ and $\alpha 1\beta 1$ integrin collagen receptors suggested that these two integrins control collagen deposition in the extracellular matrix and fibroblast-mediated matrix contraction (Schiro *et al.*, 1991; Langholz *et al.*, 1995; Riikonen *et al.*, 1995; Gardner *et al.*, 1999; Ivaska *et al.*, 1999; Ravanti *et al.*, 1999). Therefore, the dermal matrix of the wounded animals was evaluated on days 7, 14, and 21 after wounding. As shown in Figure 1c, there was no discernible morphological difference in the dermal collagen when H&E and Gomori's trichrome stained 6 mm² excisional wounds were evaluated. Although no detectable changes in collagen deposition were noted morphologically, collagen fibrillogenesis was not evaluated and our data do not exclude that there may be differences in collagen fibrillogenesis between wild-type and $\alpha 2$ -null mice that are only detectable at the electron microscopic level. Future studies will focus on collagen structure using transmission electron microscopy. Evaluation of incisional biopsies at the same time points also failed to identify a difference in the dermal extracellular matrix (data not shown).

Total collagen deposition into polyvinyl alcohol sponges, placed beneath the skin of the back, was quantitated on days 7, 10, 14, and 21 post-implantation. As shown in Figure 2a,

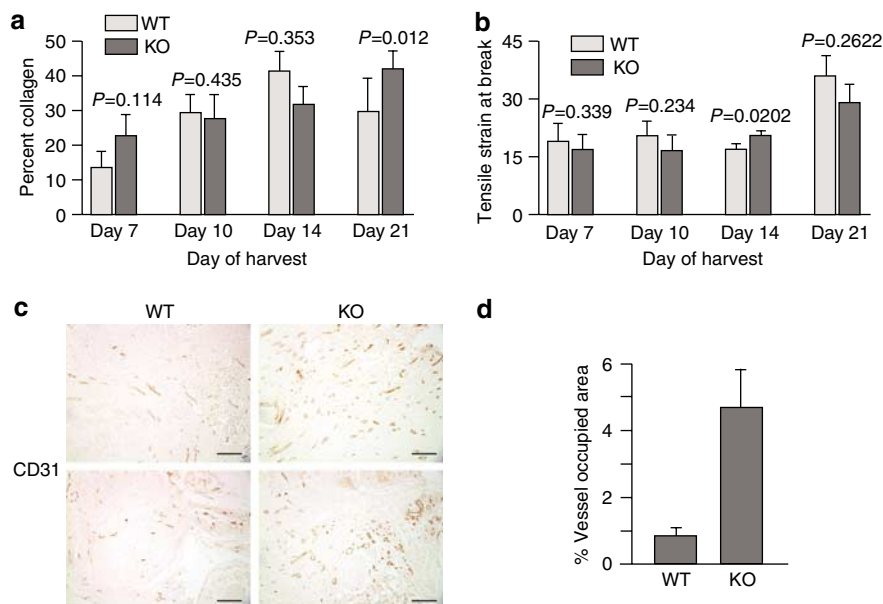


Figure 2. Neangiogenesis but not collagen deposition nor tensile strength are altered in $\alpha 2$ -null mice. (a) Total collagen deposition into polyvinyl alcohol sponges on days 7, 10, 14, and 21. Data are presented as the mean and SEM (6–10 animals per genotype). (b) Tensile strain of incisional wounds was determined at days 7, 10, 14, and 21. Data are presented as the mean and SEM (6–10 animals per genotype). (c) Immunohistochemical analysis with anti-CD31 staining of 6 mm² excisional wounds from day 10 after wounding wild-type (+/+) and $\alpha 2$ -null (–/–) animals (bar = 100 μ m) (original magnification \times 200). (d) Total area occupied by CD31-positive structures representing vascular area as a percentage of total dermal area ($*P<0.05$) on day 10 after wounding. Data are presented as the mean and SEM (six animals per genotype).

no differences in the amount of collagen deposition in the sponges at days 7, 10, or 14 were observed between the wild-type and $\alpha 2$ -null mice. However, at day 21, the percentage of collagen within the sponges of $\alpha 2$ -null mice was slightly and significantly increased ($P=0.012$). In this model, the $\alpha 2\beta 1$ integrin did not appear to influence collagen accumulation. The tensile properties of a wound depend not only on the amount of collagen, but also on the organization and crosslinking of the matrix. The biomechanical properties of incisional wounds were determined at days 7, 10, 14, and 21 using the parameters of tensile strength, elastic modulus, breaking energy, and tensile stain. The parameters of tensile strength, elastic modulus, breaking energy were not significantly different between wild-type and $\alpha 2$ -null mice at any time point (data not shown). As shown in Figure 2b, there was higher tensile strain in wounds from $\alpha 2$ -null mice at day 14, and a somewhat lower level in wounds of $\alpha 2$ -null mice, relative to wild-type animals at day 21.

To further evaluate the marked difference in neoangiogenesis observed in H&E-stained sections of wounded wild-type and the $\alpha 2$ -null skin, the area occupied by vessels within and around the granulation tissue beneath the wounded area was evaluated by immunohistochemical staining with anti-CD31 antibody. Anti-CD31 staining accentuated the vessels and highlighted the marked increase in the vessel area in the $\alpha 2$ -null animals. As shown in Figure 2c, at day 10 after wounding the vascular area was significantly increased and the vessels appeared larger in the $\alpha 2$ -null compared to wild-type animals. Quantitative analysis revealed a significant 5-fold increase in the total area occupied by vessels in $\alpha 2$ -null mice compared to wild-type mice at day 10 (Figure 2d). A similar increase in vascular area was seen between days 3 and 14 (data not shown).

In summary, deficiency of the $\alpha 2\beta 1$ integrin resulted in only subtle differences in wound repair with a slightly more rapid pace of re-epithelialization at day 6. There was no discernible difference in collagen deposition or dermal matrix reorganization to cause changes in tensile strength. The major observed difference in wound healing between wild-type and $\alpha 2$ -null mice was the increased neoangiogenesis in the $\alpha 2$ -null mice.

$\alpha 2\beta 1$ integrin-dependent keratinocyte adhesion

To define further the functional role of the $\alpha 2\beta 1$ integrin on keratinocytes, primary keratinocytes were isolated from wild-type and $\alpha 2\beta 1$ integrin-deficient mice. Flow cytometric analysis of primary keratinocytes from wild-type animals revealed high level expression of the $\alpha 2\beta 1$ integrin, as shown in Figure 3a. As expected, $\alpha 2$ -deficient keratinocytes did not express the integrin (Figure 3a). As the $\alpha 2\beta 1$ integrin binds preferentially to type I collagen, the ability of wild-type and $\alpha 2$ -deficient primary keratinocytes to adhere to either type I collagen or fibronectin was evaluated in the absence of serum. As expected wild-type keratinocytes adhered to type I collagen in a Mg^{2+} -dependent and EDTA-inhibitable manner. In contrast, $\alpha 2$ -deficient keratinocytes failed to effectively adhere to type I collagen in 1-hour assays (Figure 3b). Both wild-type and $\alpha 2$ -null keratinocytes effectively adhered to

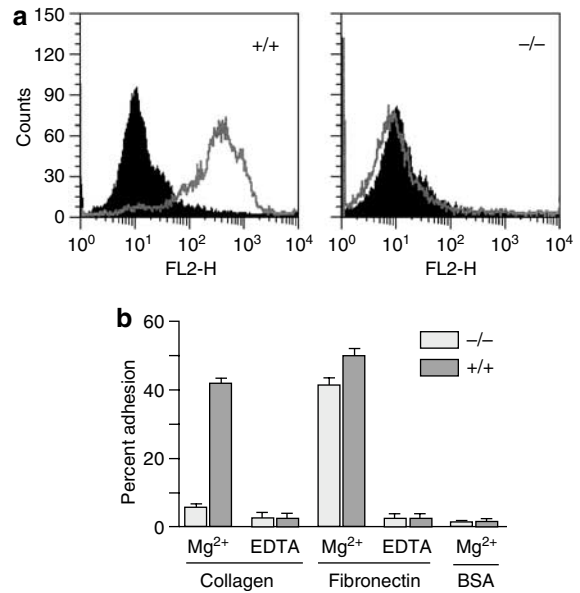


Figure 3. Primary keratinocytes from wild-type and $\alpha 2$ -null mice. (a) Flow cytometric analysis of primary keratinocytes isolated from wild-type (+/+) or $\alpha 2$ -deficient (-/-) animals using a fluorescent anti- $\alpha 2$ integrin antibody (open curves). The solid curves represent unlabeled control cells. (b) Adhesion of wild-type and $\alpha 2$ -null keratinocytes plated on type I collagen, fibronectin, or BSA in the presence of either Mg^{2+} or EDTA.

fibronectin in a similar manner suggesting that other integrins that mediate adhesion to fibronectin were not altered in $\alpha 2$ -null mice (Figure 3b).

$\alpha 2\beta 1$ integrin-dependent migration

As re-epithelialization requires keratinocyte migration across the wounded dermis, the ability of $\alpha 2$ -deficient keratinocytes to migrate on a monolayer of type I collagen or fibronectin was evaluated. As primary murine keratinocytes do not survive overnight in serum-free media used in adhesion assays, wild-type and $\alpha 2$ -deficient keratinocytes were cultured in 10 mm cloning cylinders in keratinocyte culture media containing 5% calcium-chelated fetal bovine serum at a density of 1×10^6 cells for 24 hours. As shown above, the $\alpha 2$ -deficient keratinocytes did not adhere to collagen in 1 h in the absence of serum, but a monolayer of adherent wild-type and $\alpha 2$ -deficient cells was generated after 24 hours using this approach. A 1 mm scratch wound was then made across the cell monolayer. Keratinocyte migration across the denuded matrix was observed, photographed, and subsequently quantitated 5, 10, and 24 hours after wounding. As shown in Figure 4, wild-type keratinocytes migrated on both type I collagen (Figure 4a and b) and fibronectin (Figure 4c) and completely closed the *in vitro* wounds by 24 hours. In contrast, migration of $\alpha 2$ -null keratinocytes on type I collagen was significantly impaired and 40% of the scratch wound remained free of cells at 24 hours (Figure 4a and b). Migration of $\alpha 2$ -null keratinocytes on fibronectin was similar to that of wild-type cells (Figure 4c). Therefore, $\alpha 2$ -deficient keratinocyte migration on collagen is impaired but not completely eliminated in the presence of low concentrations of serum. It

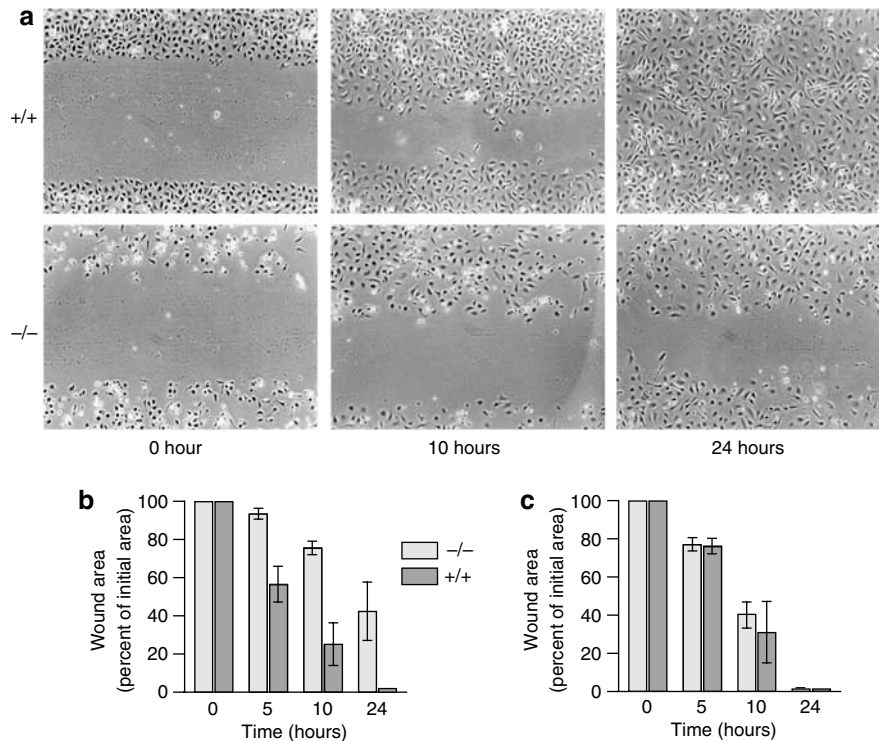


Figure 4. Migration of wild-type and $\alpha 2$ -null keratinocytes plated on type I collagen or fibronectin. (a) Photomicrographs of wild-type and $\alpha 2$ -null keratinocyte scrape wounds plated on type I collagen and photographed at 0, 10, and 24 hours after wounding; (b) quantitative migration of wild-type and $\alpha 2$ -null keratinocytes plated on type I collagen; (c) quantitative migration of wild-type and $\alpha 2$ -null keratinocytes plated on fibronectin.

is likely that low concentrations of fibronectin and vitronectin in the serum provide, in part, a matrix for migration for the $\alpha 2$ -null keratinocytes.

$\alpha 2\beta 1$ integrin-independent *ex vivo* migration

The impaired ability of $\alpha 2$ -deficient keratinocytes to adhere to and migrate on type I collagen substrates *in vitro* suggested that $\alpha 2\beta 1$ -deficient mice should have displayed a defect in epithelial development and wound repair that was not observed *in vivo*. To explore further these *in vivo* and *in vitro* differences, 4 mm diameter punch biopsies from the dorsal surface of wild-type or $\alpha 2$ -deficient mice were placed into *ex vivo* culture and the ability of the wild-type and $\alpha 2$ -deficient epidermal sheets to migrate over the exposed dermis was evaluated. Wild-type and $\alpha 2$ -null epithelia migrated over the exposed dermal surface of the explanted tissue in a similar manner (Figure 5). In addition, migration of the epidermal sheet was not inhibited by the addition of inhibitory antibodies directed against either the $\alpha 2$ or $\alpha 1$ integrin subunit (data not shown). These data suggest several possibilities. One possibility is that the $\alpha 2\beta 1$ integrin plays no role in wound healing. A second more likely possibility is that the $\alpha 2\beta 1$ integrin plays a dramatically different role when keratinocytes are exposed to a complex matrix containing multiple collagens, laminins, fibronectin, and other matrix components than when they are exposed to a matrix composed predominantly of type I collagen. Third, "compensatory" changes in gene expression that occur in the

absence of the $\alpha 2\beta 1$ integrin might contribute to the normal wound repair. We therefore evaluated the consequences of altered $\alpha 2\beta 1$ integrin expression on the expression of other adhesion receptors and molecules required for wound repair, such as the MMPs.

$\alpha 2\beta 1$ integrin-dependent gene expression

The expression of other integrin subunits and members of the MMP family, molecules known to be relevant to cell adhesion, migration, and wound healing, by wild-type and $\alpha 2$ -deficient primary keratinocytes adherent to either type I collagen or fibronectin for 48 hours was determined by quantitative real-time PCR (qRT-PCR). Expression of genes by $\alpha 2$ -null keratinocytes relative to expression by wild-type cells is shown in Figure 6. Expression of the $\alpha 1$ and $\beta 3$ integrin subunit genes and MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13, and COLA genes was increased (greater than 2.5-fold) by $\alpha 2$ -null keratinocytes relative to the levels of expression exhibited by wild-type cells plated on type I collagen (Figure 6a). Expression of the MT1-MMP gene was increased in $\alpha 2$ -null keratinocytes but not 2.5-fold. The expression of the $\beta 4$ integrin subunit by $\alpha 2$ -deficient keratinocytes was extremely variable (Figure 6a). Surprisingly, expression of many genes including those encoding the $\alpha 3$, $\alpha 5$, $\alpha 6$, αv , $\beta 1$, and $\beta 5$ integrin subunits was similar when $\alpha 2$ -null and wild-type keratinocytes were plated on type I collagen for 48 hours (Figure 6a). The differences in gene expression of the integrin receptor subunits and MMPs were

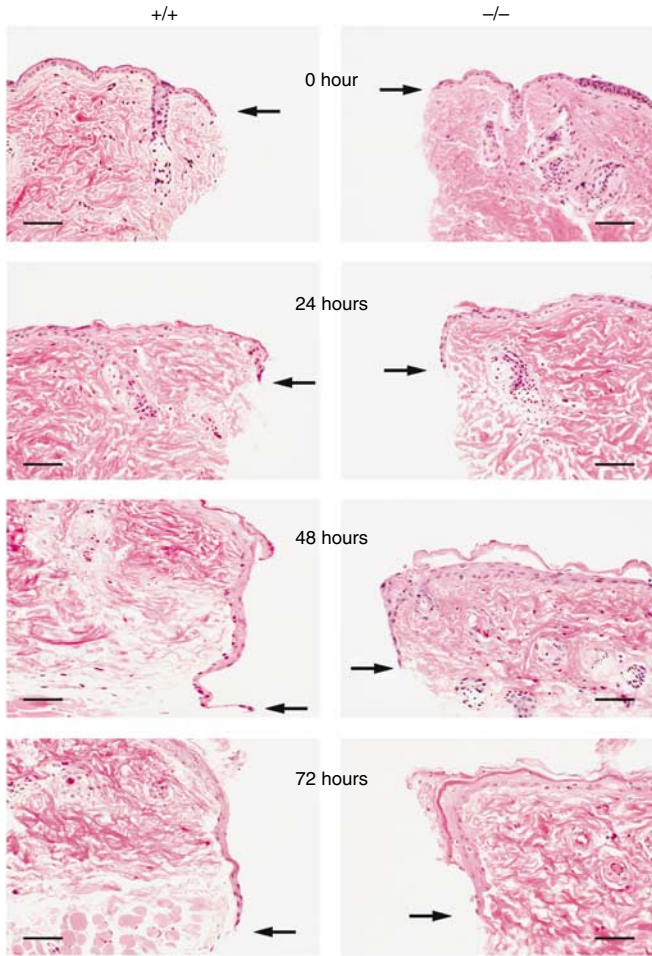


Figure 5. Migration of epidermal keratinocytes over the dermal edge of explanted skin obtained from wild-type and $\alpha 2$ -null mice. The arrows indicate the location of the leading edge of keratinocytes at 0, 24, 48, and 72 hours (bar = 100 μm) (original magnification $\times 200$).

much less dramatic when wild-type keratinocytes and $\alpha 2$ -null keratinocytes were plated on fibronectin (Figure 6b). The increased levels of MMP-8 messenger RNA and the slightly increased levels of MMP-3 and McolA demonstrated by $\alpha 2$ -null keratinocytes cultured on collagen were also observed when the cells were cultured on fibronectin. However, the relative increase in these two MMPs was less pronounced than that observed when the cells were plated on collagen. The only gene evaluated whose expression was slightly diminished by the absence of the $\alpha 2\beta 1$ integrin when keratinocytes were cultured on either collagen or fibronectin was the $\beta 4$ integrin subunit. In fact, expression of the $\beta 4$ integrin subunit by $\alpha 2$ -deficient keratinocytes was decreased relative to expression by wild-type keratinocytes plated on either type I collagen or fibronectin. In large part, the changes in gene expression observed in the absence of the $\alpha 2\beta 1$ integrin were ligation-dependent and resulted from the interaction, or lack thereof, of the $\alpha 2\beta 1$ integrin with type I collagen. In contrast, diminished expression of the $\beta 4$ integrin subunit gene was independent of integrin ligation.

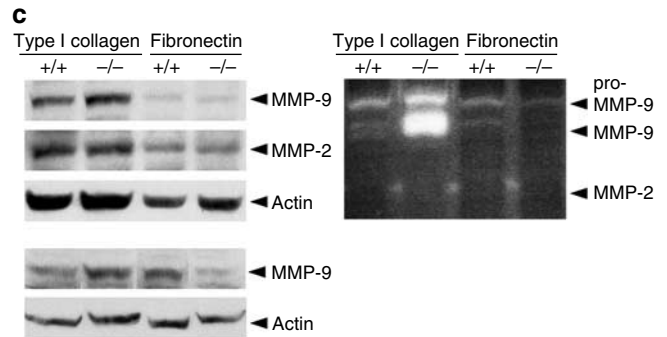
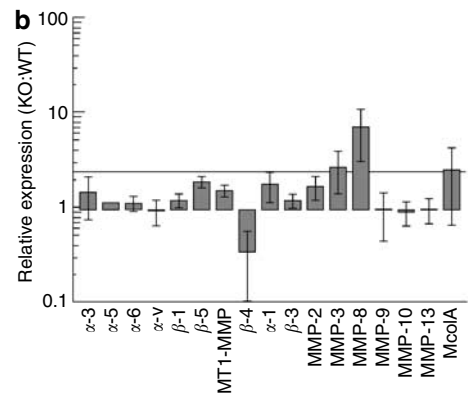
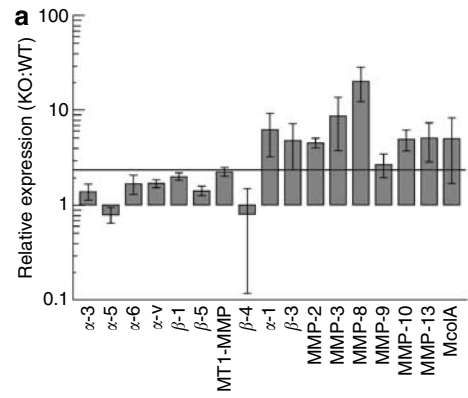


Figure 6. Altered gene expression in $\alpha 2$ -null keratinocytes. Gene expression determined by qRT-PCR using RNA from wild-type and $\alpha 2$ -null keratinocytes plated on either (a) type I collagen or (b) fibronectin. Bars represent transcript expression in $\alpha 2$ -null cells relative to wild-type cells and normalized to expression of a glyceraldehyde-3-phosphate dehydrogenase control gene. Results are the averages of three separate experiments. (c) Protein expression and activity of MMP-2 and MMP-9 determined by immunoblot analysis and gelatin zymography and protein expression of MMP-8 determined by immunoblot analysis.

Changes in the expression of messenger RNA in cells and tissues may or may not be reflected by altered protein expression. Therefore, the changes detected by qRT-PCR were evaluated by Western blot analysis when antibodies were available. As shown in Figure 6c, expression levels of MMP-9 were increased 2-fold or greater when wild-type or $\alpha 2$ -null keratinocytes were plated on type I collagen in comparison to fibronectin. In addition, $\alpha 2$ -null keratinocytes expressed slightly higher levels of MMP-9 than wild-type keratinocytes when plated on type I collagen. Expression

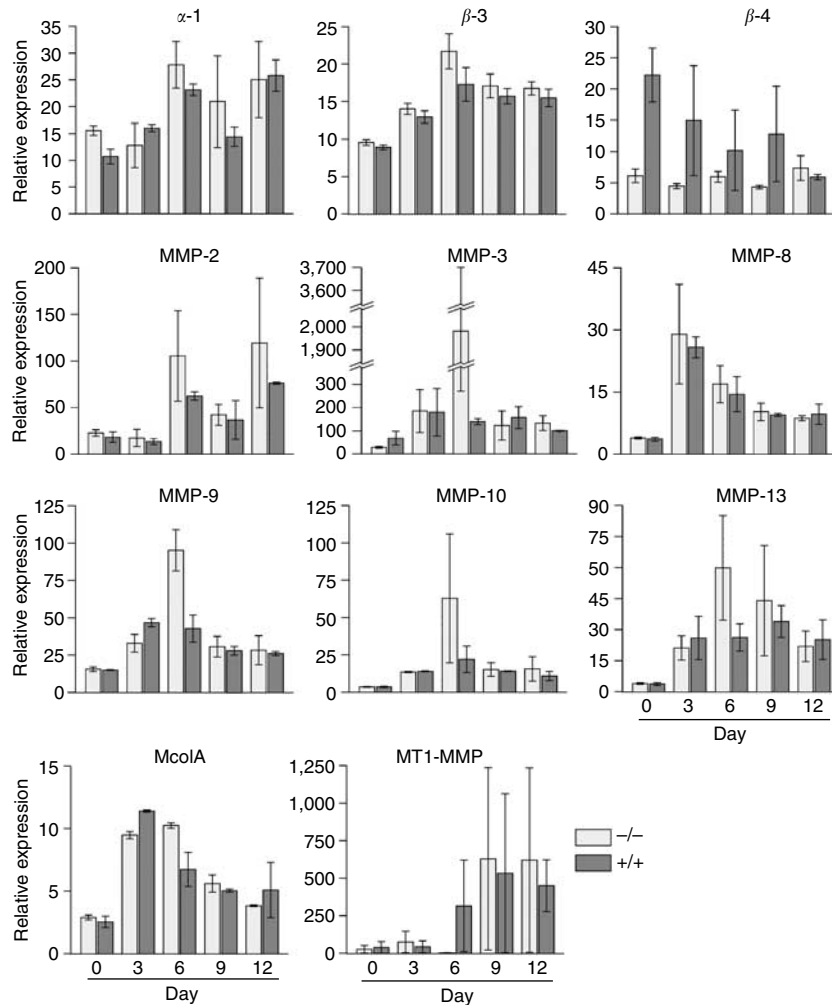


Figure 7. Gene expression determined by qRT-PCR using RNA isolated from unwounded skin and cutaneous wounds from wild-type and $\alpha 2$ -null animals before wounding and on post-wound days 3, 6, 9, and 12. Bars represent transcript expression in wounded skin relative to expression of glyceraldehyde-3-phosphate dehydrogenase in the same sample. Results are the averages of two separate experiments with three animals in each experiment.

of MMP-2 was slightly increased by both wild-type and $\alpha 2$ -deficient keratinocytes plated on collagen but not on fibronectin. There were no detectable differences in MMP-2 expression between wild-type and $\alpha 2$ -deficient keratinocytes. The expression of MMP-8 was more variable (Figure 6d). MMP-8 was expressed at higher levels by wild-type keratinocytes plated on fibronectin and by $\alpha 2$ -null keratinocytes plated on collagen. The $\alpha 2$ -null cells expressed lower levels of MMP-8 when plated on fibronectin. To assess the activity of MMP-2 and MMP-9 gelatin zymography was performed (Figure 6e). Zymography confirmed the increased expression of MMP-9 when $\alpha 2$ -deficient keratinocytes were plated on collagen in comparison to wild-type cells plated on collagen or either wild-type or $\alpha 2$ -deficient keratinocytes plated on fibronectin. MMP-2 was only detected at extremely low levels by gelatin zymography.

Gene expression in wounded tissue

The marked discordance in the role of the $\alpha 2\beta 1$ integrin in keratinocyte adhesion and migration *in vitro* on defined substrates versus *in vivo* on a complex matrix suggested

either that changes in gene expression were more a consequence of the *in vitro* culture conditions or that the alterations in gene expression by $\alpha 2$ -null keratinocytes compensated for the lack of the integrin and allowed normal wound healing to proceed. To evaluate these two alternatives, expression of several genes that were altered *in vitro* was determined in wounded tissue *in vivo*. Biopsies were harvested from wounded animals at various time points after the induction of a wound and gene expression was evaluated by qRT-PCR (Figure 7). In the resting, uninjured skin, (time=0), the expression of the $\alpha 1$ integrin subunit was slightly elevated and the expression of the $\beta 4$ subunit was diminished in the $\alpha 2$ -null versus the wild-type mouse. Expression of all other integrin subunits and the MMP's was similar in the unwounded wild-type and the $\alpha 2$ -null skin.

In contrast to the expression in uninjured skin, the temporal changes in gene expression following injury were different in the wild-type and $\alpha 2$ -null mouse and resembled the differences in expression observed in primary keratinocytes plated on type I collagen. Expression of MMP-2, MMP-9, MMP-10, and MMP-13 messenger RNA from biopsies of

$\alpha 2$ -null and wild-type wounded skin increased in a time-dependent manner with a peak occurring between days 3 and 6, as shown in Figure 7. Following the peak there was a gradual decline to baseline levels by day 12, except for MMP-2. Increased expression for all MMPs was relatively greater in the $\alpha 2$ -null than in the wild-type wounds. Although expression of the $\alpha 1$ integrin subunit was greater in unwounded $\alpha 2$ -null than wild-type skin, the difference in $\alpha 1$ integrin subunit expression was no longer evident after wounding.

MMP-2 messenger RNA was increased in both genotypes by post-wound day 6. Expression of MMP-2 was 1.7 times greater on post-wound day 6 and 1.6 times higher on post-wound day 12 in $\alpha 2$ -null mice compared to wild-type animals. MMP-2 levels did not taper by day 12 in wounds from either genotype. MMP-9 expression was maximal between post-wound days 3 and 6 in wild-type animals and gradually declined to near baseline levels by post-wound day 12. In the $\alpha 2$ -null mouse, MMP-9 expression reached a peak 2.2 times higher than that observed for wild-type animals on post-wound day 6. MMP-9 expression returned to near baseline levels by post-wound day 12. Similarly, expression of MMP-10 and MMP-13 peaked between post-wound days 6–9 in both genotypes; on day 6, expression of MMP-10 was 2.9 times, and MMP-13 was 2.3 times higher in $\alpha 2$ -null than wild-type wounds. MMP-3 messenger RNA levels were higher in the $\alpha 2$ -null mouse wound only on day 6 after wounding. No differences were noted in the levels of expression of the $\beta 3$ integrin subunit, MMP-8, MT1-MMP, and COLA.

DISCUSSION

In our earlier report we presented initial data that lack of the $\alpha 2\beta 1$ integrin does not alter normal epithelial development and differentiation, nor does it affect the rate of re-epithelialization of a 100 mm² excision. In this report we now compare multiple parameters of wound healing in wild-type and $\alpha 2\beta 1$ integrin-deficient mice. In fact, genetic deletion of the $\alpha 2\beta 1$ integrin does not alter the rate of re-epithelialization, the inflammatory component of granulation tissue, the quantity of collagen deposited within the wound, or the tensile strength of the wound in the murine model. In contrast to the many similarities between the wild-type and the $\alpha 2$ -null animals, deletion of the $\alpha 2\beta 1$ integrin resulted in a dramatic increase in neoangiogenesis in the wounded environment.

In contrast to the dispensable role of the $\alpha 2\beta 1$ integrin expression plays in re-epithelialization *in vivo*, we observed that $\alpha 2$ -deficient primary keratinocytes adhere poorly to type I collagen *in vitro* and display impaired migration across type I collagen substrates. The inability to migrate on type I collagen is consistent with earlier reports by other investigators demonstrating that the $\alpha 2\beta 1$ integrin regulates expression and localization of MMP-1 at the migration front during wound healing of human epithelium (Pilcher *et al.*, 1997). Although $\alpha 2$ -null keratinocytes were unable to migrate effectively across a type I collagen matrix *in vitro*, they were able to migrate over the complex and heterogeneous matrix of the dermis in *ex vivo* experiments. Collectively these data

suggest that *in vivo* wound healing in mice is not dependent on the interaction between the $\alpha 2\beta 1$ integrin and collagen.

The changes in gene expression that occur downstream of $\alpha 2\beta 1$ integrin expression and/or ligation suggest that $\alpha 2\beta 1$ integrin expression is a determinant of the expression of numerous genes of the MMP and integrin families, as well as other genes contributing to extracellular matrix composition and remodeling. Specifically, greater than 2.5-fold increases in the expression of the $\alpha 1$ and $\beta 3$ integrin subunits and MMP-2, MMP-9, MMP-10, and MMP-13 were observed when $\alpha 2$ -null versus wild-type keratinocytes were plated on type I collagen. Lesser increases in expression of the same molecules were observed in $\alpha 2$ -null keratinocytes cultured on fibronectin, suggesting that both expression and subsequent ligation of the $\alpha 2\beta 1$ integrin by collagen contributed to the observed differences. On the other hand, expression of MMP-3, MMP-8, and COLA was upregulated greater than 2.5-fold by $\alpha 2$ -null cells on either substrate. The absence of the $\alpha 2\beta 1$ integrin may remove a signaling pathway that suppresses these substrate-independent target genes. However, a more likely possibility is that the compensatory amplification of other matrix signaling pathways led to higher expression of these three target genes. Furthermore, the observed changes in gene expression of matrix molecules, adhesion receptors, and MMPs that occur as a consequence of the loss of expression of the $\alpha 2\beta 1$ integrin and/or ligation of the $\alpha 2\beta 1$ integrin suggest that these changes may contribute to the normal wound healing observed in the $\alpha 2\beta 1$ integrin-deficient mouse. The changes in gene expression were observed not only in cell culture models but also temporally during wound repair *in vivo*.

The marked increase in neoangiogenesis in the wounding environment is intriguing. The results reported in this manuscript provide evidence that lack of $\alpha 2\beta 1$ integrin does not effect developmental angiogenesis but has a significant effect on pathologic angiogenesis in other microenvironments including wound healing.

The correlation between lack of $\alpha 2\beta 1$ integrin expression and increased expression of several MMPs suggests that $\alpha 2\beta 1$ integrin expression and/or ligation may negatively regulate the levels of specific MMPs. This correlation provides another potential mechanism by which the $\alpha 2\beta 1$ integrin may regulate angiogenesis. MMP expression, particularly MMP-2 and MMP-9 are essential for angiogenesis. In addition to gene expression, MMP-9 protein was only detected in keratinocytes plated on collagen, but not on fibronectin. There is low-level MMP-9 activity by wild-type keratinocytes plated on collagen, but significant activity by $\alpha 2$ -null keratinocytes. The difference in MMP-9 activity between wild-type and $\alpha 2$ -null cells is intriguing and suggests that other, as yet unidentified factors controlled by the integrin play a role in regulating activity. Alternatively, loss of the $\alpha 2\beta 1$ integrin may result in the loss of modulation of positive signals from other integrins such as the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins. Markedly reduced or absent expression of the $\alpha 2\beta 1$ integrin is observed in poorly differentiated carcinomas during tumor progression (Pignatelli *et al.*, 1990, 1991, 1992; Zutter *et al.*, 1990, 1993; Koukoulis *et al.*, 1991). Our findings suggest that loss of the

$\alpha 2\beta 1$ integrin may be a determinant of the increased MMP expression associated with tumor progression and neoangiogenesis, invasion, and metastasis.

Many of the changes in keratinocytes observed in the $\alpha 2$ -null mouse were also observed in mice with targeted deletion of the $\beta 1$ integrin, suggesting that several of the defects observed in the $\beta 1$ integrin-targeted mice are a consequence of the loss of the $\alpha 2\beta 1$ integrin. Both $\alpha 2$ -null and $\beta 1$ -null keratinocytes demonstrated poor adhesion to collagens and diminished cell migration. The alterations in the expression of several genes regulated during wound healing, including MMP-10 and MMP-13, were observed in wounded tissue from both the $\alpha 2$ -null and $\beta 1$ -null mice (Grose *et al.*, 2002). However, other defects observed in the $\beta 1$ -null mouse were not apparent in the $\alpha 2$ -null mouse. For example, wound healing was severely impaired owing to failure of re-epithelialization in the $\beta 1$ -null mouse, but not in the $\alpha 2$ -null mouse indicating that α subunits other than the $\alpha 2$ integrin subunit were responsible for the wounding defect observed in the absence of the $\beta 1$ integrin. The most dramatic difference in MMP expression between wild-type and $\alpha 2$ -null mice *in vivo* was noted for MMP-3, or stromelysin, 6 days after wounding. MMP-3 is known to be upregulated within 24 hours in the wounded microenvironment. Expression of MMP-3 in non-malignant tissue is primarily by stromal cells, and not by the epithelial cells. Although deletion of MMP-3 had a no impact on reepithelialization, the MMP-3-null mice healed more slowly and failed to contract their wounds (Bullard *et al.*, 1999). One could postulate that the relatively normal wound closure observed in the $\alpha 2$ -null mouse could be due to compensatory upregulation of MMP-3.

A decrease in the $\beta 4$ integrin subunit was observed in both the $\alpha 2$ -null mouse and the $\beta 1$ -null mouse. In the $\beta 1$ -null epidermis, the hemidesmosomal disruption with disorganized $\beta 4$ integrin localization along the basal cell layer suggested decreased levels of $\beta 4$ integrin expression. In the $\alpha 2$ -null mouse, the level of $\beta 4$ integrin expression was dramatically decreased in primary keratinocytes and in the wounded and unwounded skin of the $\alpha 2$ -null animals. The decreased expression of the $\beta 4$ integrin subunit in $\alpha 2$ -null keratinocytes is consistent with our earlier *in vitro* studies of a poorly differentiated murine mammary carcinoma cell line (Sun *et al.*, 1998). In these earlier studies, re-expression of the $\alpha 2\beta 1$ integrin restored epithelial differentiation that was accompanied by increased $\alpha 6\beta 4$ integrin expression. The present studies as well as our earlier studies indicate that $\alpha 2\beta 1$ integrin expression is an important determinant of $\alpha 6\beta 4$ integrin expression. It is noteworthy that neither the $\alpha 2$ -null nor the $\beta 1$ -null mouse exhibited the severe blistering disorder exhibited by the $\alpha 6\beta 4$ -null and the $\alpha 6$ -null mice (Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996). These findings suggest that the reduced level of the $\alpha 6\beta 4$ integrin exhibited by either the $\alpha 2$ -null or the $\beta 1$ -null mice is sufficient to prevent the blistering phenotype.

In summary, although wound healing is remarkably normal in the absence of $\alpha 2\beta 1$ integrin expression, mice lacking $\alpha 2\beta 1$ integrin expression exhibit increased angiogenesis in the wounding microenvironment. It now appears

that neoangiogenesis stimulated by not only the tumor microenvironment, but in addition wounding, is regulated in part by $\alpha 2\beta 1$ integrin expression. Although $\alpha 2$ -deficient primary keratinocytes adhered poorly to type I collagen *in vitro* and displayed impaired migration across a matrix of type I collagen *in vitro*, $\alpha 2$ -null keratinocytes are able to migrate over the heterogeneous and complex matrix that is contained within the dermis as shown in the *ex vivo* migration data reported here. Our data from the murine model suggest that *in vivo* wound healing does not require the $\alpha 2\beta 1$ integrin. In addition, the changes in gene expression suggested that other matrix molecules, other receptors and the upregulation of MMP's could potentially account for normal wound healing in the $\alpha 2$ -null mouse. *In vitro* studies of human skin have suggested that the $\alpha 2\beta 1$ integrin is required for wound healing (Koukoulis *et al.*, 1991; Pignatelli *et al.*, 1992). Inhibitory antibodies against the $\alpha 2\beta 1$ integrin prevent keratinocyte migration on type I collagen and across wounded dermis and block fibroblast contraction of three-dimensional collagen gels (Koukoulis *et al.*, 1991; Pignatelli *et al.*, 1992). However, deficiency of the $\alpha 2\beta 1$ integrin failed to alter wound healing *in vivo* and had no effect on the migration of keratinocytes over exposed dermis suggesting that $\alpha 2\beta 1$ integrin does not play an obligatory role in wound healing. There are several possible explanations for the discrepancy between the deletion of the gene and antibody inhibition studies with human cells. One arises from the use of two different organisms that may not be mechanistically equivalent as model systems. In contrast to murine skin, which is rich in loose connective tissue, the human dermis is composed of dense fibrillar collagens. As human keratinocytes must migrate across a collagen-rich substratum, perhaps the human $\alpha 2\beta 1$ integrin has evolved additional functions not required in the murine models. In addition, specific MMPs and their matrix specificity differ between humans and mice. In the human MMP-1 is expressed by keratinocytes at the migrating front of the epithelial sheet during wound closures. In mice, MMP-13 is expressed by the same cell population, but, MMP-13 and MMP-1 have distinct substrate specificities and distinct functions. Therefore, the difference between not only the integrin, but the matrix and the MMP specificity may explain the dramatic differences between the importance of the $\alpha 2\beta 1$ integrin in humans and mice. Second, on binding the integrin, inhibitory antibodies may stimulate signals downstream of the integrin that are responsible for the observed phenotype. Thus, antibodies could produce effects that differ from those because of lack of integrin expression. Finally, the major impact on wound healing that results from deleting the $\alpha 2\beta 1$ integrin is on neoangiogenesis. The results suggest that the integrin controls neoangiogenesis in a number of models including wound healing and within the tumor microenvironment.

MATERIALS AND METHODS

Mice

The development of mice with complete genetic deletion of the $\alpha 2$ integrin subunit gene was previously described (Chen *et al.*, 2002). $\alpha 2$ Integrin subunit-deficient mice were backcrossed 10 times onto

the C57/Bl6 background to obtain animals that were 99% genetically C57/Bl6. The animals were housed in pathogen-free conditions at Vanderbilt University Medical Center, in compliance with IACUC regulations. Animals were used at 6–12 weeks of age. Within individual experiments, mice were appropriately age and sex matched.

In vivo wounding and tissue preparation

Six- to 12-week-old wild-type and $\alpha 2$ -null animals were anesthetized with a 0.1 ml intraperitoneal injection of ketamine (17.4 mg/ml) and xylazine (2.6 mg/ml). The dorsal surface of the animal was shaved and sterilized. Full-thickness wounds were made in the flank using a disposable, sterile, 6-mm punch biopsy (Miltex Instruments, Bethpage, NY). Animals were housed in individual cages with a wire grating placed above the bedding to prevent it from becoming embedded in the wounds. For quantitation, photographic wound closure data were collected using a Coolpix 5000 digital camera (Nikon, Tokyo, Japan) and wound area was measured using Image-Pro Software (Mediacybernetics Inc., Silver Spring, MD). Closure area was quantified against a scale bar photo label in each image. Means per group and SEM were calculated and graphed. Wounds were allowed to heal for varying lengths of time at which point animals were killed and the wounds completely excised. The excised wounds were immediately homogenized in Trizol (Life Technologies, Rockville, MD) for isolation of total RNA.

Histology, immunohistochemistry, and immunofluorescence analyses

Morphology of the wounds was initially evaluated on paraffin-embedded, H&E, or Gomori's trichrome-stained sections. Immunohistochemical analyses were carried out on 6 μ m frozen-sections, fixed briefly in acetone, and stained with the rat anti-CD31 antibody (BD Biosciences, Pharmingen, San Diego, CA) at a 1:100 dilution. The signal was detected with biotinylated goat-antirat IgG and avidin-biotin complex reagent, the VECTASTAIN Elite ABC Kit (Vector Labs, Burlingame, CA). The slides were examined and photographed using a Nikon 80i manual system microscope with motorized shutter for digital imaging and images were processed with Metamorph imaging system (Molecular Devices, Sunnyvale, CA).

Wound analysis

Collagen content of wounds was determined from the acid-hydrolyzed homogenate of a segment of each implanted polyvinyl alcohol sponge. Amino-acid analysis of the derivatized hydrolysate yielded hydroxyproline and other amino acids to estimate both total and relative collagen content (Buckley *et al.*, 1988). Mechanical testing of incisional wounds was accomplished by analysis of tissue strips that were excised post mortem to include a transverse segment of a 1 cm linear incision. After storage at -20°C within saline-moistened gauze in an air-tight wrapping, skin strips were thawed and tested while moistened in an Instron biomechanical testing device, using three preconditioning cycles and an elongation rate of 1 mm/s. Bluehill™ software was used to collect data on tensile strength, elastic modulus, breaking energy, and tensile stress.

Primary keratinocyte isolation, culture, and characterization

Primary keratinocytes were isolated from the skin of neonatal mice as described previously (Yuspa *et al.*, 1980). Skin from the torso of

one to 2-day old mice was exposed to 0.25% trypsin in Hank's-buffered saline solution overnight at 4°C . The epidermis was removed as a sheet, minced, and then agitated at 37°C for 45 minutes in 10 ml of keratinocyte growth media. The minced epidermis was filtered through a 70 μ m cell strainer (BD Biosciences Discovery Labware, Bedford, MA) to remove the stratum corneum. Cells were pelleted by centrifugation and resuspended in fresh keratinocyte growth media for experiments. Keratinocyte growth media was composed of Minimum Essential Medium modified for suspension cultures (Invitrogen, Carlsbad, CA) supplemented with 5% calcium-chelated fetal bovine serum (HyClone, Logan, UT), 0.05 mM CaCl_2 , 4 mM glutamine (Invitrogen), 5 $\mu\text{g/ml}$ insulin (Sigma-Aldrich, St Louis, MO), 10 ng/ml epidermal growth factor (Invitrogen), 0.4 $\mu\text{g/ml}$ hydrocortisone (Sigma-Aldrich), 10^{-10} M cholera toxin (Sigma-Aldrich), 2×10^{-9} M triiodothyronine (Sigma-Aldrich), 100 IU/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin (Invitrogen). Chelated fetal bovine serum was prepared using Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA) in accordance with the manufacturer's instructions.

Flow cytometric analysis was performed on freshly isolated cells harvested with 2 mmol/l EDTA in phosphate-buffered saline (pH 7.45). Single cells (1×10^6) in phosphate-buffered saline containing 1.5% horse serum were incubated with fluorescein-conjugated anti-murine $\alpha 2$ integrin antibodies (Pharmingen, San Diego, CA) for 45 minutes at 4°C . Cells were washed three times, resuspended in phosphate-buffered saline, and analyzed using a FACScan instrument (Becton Dickinson, Mountain View, CA).

Adhesion assays

Primary keratinocytes were resuspended in Hank's-buffered saline solution with either 2 mM MgCl_2 or 2 mM EDTA and plated at a density of 2×10^5 cells into wells of a 96-well plate coated with either 25 $\mu\text{g/ml}$ type I collagen (BD Biosciences Discovery Labware), 50 $\mu\text{g/ml}$ fibronectin (Sigma-Aldrich), or 0.5% BSA (Sigma-Aldrich) and allowed to adhere for 1 h at 37°C . Non-adherent cells were removed by washing and adhesion was quantitated by spectrophotometric measurement of *p*-nitrophenol produced by enzymatic conversion from *p*-nitrophenol *N*-acetyl- β -D-glucosaminide by cellular β -hexosaminidase (Santoro *et al.*, 1994).

Cell migration analysis in vitro

Primary keratinocytes resuspended in keratinocyte growth media were plated at a density of 1×10^6 cells into 10 mm cloning cylinders (Bellco Glass, Vineland, NJ) that had been placed on six-well non-tissue culture treated cluster dishes coated with either 100 $\mu\text{g/ml}$ of type I collagen or 50 $\mu\text{g/ml}$ of fibronectin and allowed to adhere for 24 hours at 37°C . Cloning cylinders were removed, 2 ml of keratinocyte growth media were added, and 1 mm wide scrape wounds were made across the cell monolayer using a disposable pipette tip. Scrape wounds were photographed at 0, 5, 10, 24 hours, and migration was digitally quantitated (Scion Image software, Scion Corp, Frederick, MD) by measuring the surface area of the wound that remained free of adherent cells at each time point.

Cell migration analysis ex vivo

Six- to 8-week-old wild-type and $\alpha 2$ -null animals were euthanized and the dorsal skin shaved, excised, and cleaned by brief, consecutive washes in 70% ethanol, Betadine, and sterile water.

Table 1. Primer sequences used in qRT-PCR analysis

mRNA	Forward primer	Reverse primer
$\alpha 1$	CACCTTTCAAAGTCTGATGTGCGTGGCA	GCTGCCACAGCGATGTAGAGCACAT
$\alpha 3$	TGGGCAAGTCTGATGTGCGTGGCA	TCTGGGTGAAGCCGCCGCTGGT
$\alpha 5$	CACTTGGCTTCAGGGCATTTC	CAACTACACCCCCAACTCACAGG
$\alpha 6$	CTGGCTTCTCGTTTGGCTATG	TGCCTTGTGGTTAATGTAGACGT
αv	TTGGGGACGACAACCTCTGACAC	TGCGGCGGGATAGAAACGATGAG
$\beta 1$	TCTACCAAAGTAGAAAGCAGGGA	ACGATAGCTTCATTGTTGCCATTC
$\beta 3$	GCTTTGGGGCCTTCGTGGACAA	CATGGGCAAGCAGGCATTCTTCAT
$\beta 4$	CACCCACCAGGAAGTACCCAGT	GACCAGTCCATAGCAGACCTCGTA
$\beta 5$	ACGACCGCCGAGAGTTTGCCAA	GCCATCCAGGAGCCTCAGTCCAC
MMP-2	TATGGATTCTGCCCCGAGACC	ACACCACACCTTGCCATCGTT
MMP-3	TCTGGGCTATACGAGGGCAGGAGG	TGGCAGCATCGATCTTCTTCACGG
MMP-8	AGCCTGTGACCCCCACCTGAGATT	GGCCGGGCCAGAACAGAGATATG
MMP-9	TCTCGGGAAGGCTCTGCTGTT	TTGGTACTGGGAGATGTCGTGTGA
MMP-10	CTCCAAGACCTGAGACCCAGACAA	TCCAATGGGATCTGCGCCAGA
MMP-13	CTGAGCGCTGCGGTTCACTTTGAG	TGAGGGCGGGATAATCTTTGTCCA
MT1-MMP	CTGAGGGTTTCCACGGCGACAGTA	TCCAGGGCTCGGCAGAAATCAAAGT
COLA	GGCAAATGCAGCAGTTATTGGGC	ATGGGGCCACATCAGGCACCC

MMP, matrix metalloproteinase; qRT-PCR, quantitative real-time PCR.

Punch biopsies (4 mm) were removed and placed dermis side down in wells of an 18-well cluster dish. Explants were allowed to adhere for 5 minutes before adding culture medium (DMEM, Invitrogen) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin) in sufficient volume to just cover the epidermis of the explanted tissue. Explants were incubated at 37°C for up to 72 hours and media was replaced every 24 hours. Anti- $\alpha 2$ integrin subunit and anti- $\alpha 1$ integrin subunit inhibitory antibodies (BD Biosciences Pharmingen, San Diego, CA) (10 μ g/ml) were added. The quantitation of epithelial tongue migration was carried out by photographing the slides and measuring the distance from the cut margin.

RNA isolation

Total RNA from primary keratinocytes cultured on Petri dishes coated with either 100 μ g/ml of type I collagen or 50 μ g/ml of fibronectin for 48 hours was isolated using the RNeasy RNA isolation system (Qiagen, Valencia, CA) and following the manufacturer's recommendations. Total RNA from full-thickness skin wounds was isolated from samples homogenized in Trizol following the manufacturer's recommendations followed by processing using the RNeasy RNA isolation system as described above.

QRT-PCR analysis

Total RNA isolated from primary keratinocytes or wound tissue was treated with DNase and reverse transcribed to single-stranded cDNA using Superscript II (Life Technologies) with an oligo-dT primer. A single primer/probe set for each gene of interest was designed and is shown in Table 1. QRT-PCR was performed with a Bio-Rad iCycler (Bio-Rad, Hercules, CA) and the SYBR green reporter dye (Applied Biosystems, Foster City, CA). Each assay included a negative control

using RNA not subjected to reverse transcription. Expression of each gene was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase.

Immunoblot analysis

Keratinocytes were lysed in lysis buffer (50 mmol/l *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, (pH 7.2), 250 mmol/l NaCl, 2 mmol/l EDTA, 0.1% Nonidet P-40, 10 μ g/ml aprotinin, 5 μ g/ml leupeptin, 40 mmol/l NaF, 0.5 mmol/l phenylmethyl sulfonyl fluoride, 0.5 mmol/l *o*-vanadate, and 1 mmol/l dithiothreitol). Total protein concentration was determined by the Pierce protein assay (Fisher Scientific, Pittsburgh, PA). Equivalent amounts of protein lysate were subjected to SDS-PAGE and electroblotted onto Immobilon-P transfer membrane (Fisher Scientific). Immunoblots were incubated overnight with the appropriate dilution of primary antibody at 4°C followed by secondary horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hour at room temperature. Primary antibodies used included anti-murine MMP-2 (Clone 42-5011, Oncogene Inc., Cambridge, MA), MMP-8 (Biomol Inc., Plymouth Meeting, PA) and MMP-9 (Oncogene Inc., Cambridge, MA). Enhanced chemiluminescence system (Amersham Pharmacia Biotech) was used for visualization.

Zymography

Gelatin zymography was performed on cells lysed in Laemmli's sample buffer. Total protein concentration was determined as described above. One hundred micrograms of total protein was loaded on 10% SDS-polyacrylamide gels containing 0.1 mg/ml gelatin and run at 100 V for 4 hours in non-reducing conditions. After electrophoresis, gels were washed with 2.5% Triton X-100 twice for

15 minutes and incubated at 37°C in substrate buffer (50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l CaCl₂) overnight. Gels were stained with 0.5% Coomassie blue, 50% methanol, and 10% acetic acid and destained in 50% methanol, 10% acetic acid. Bands were quantified by densitometry using an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA).

Statistical analysis

Statistical analysis was performed using either analysis of variance or unpaired Student’s t-test and $P < 0.05$ was considered statistically significant. All calculations and graphs were performed using GraphPad Prism Version 4 (GraphPad Software, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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