α3β1 integrin in epidermis promotes wound angiogenesis and keratinocyte-to-endothelial-cell crosstalk through the induction of MRP3

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Summary
During cutaneous wound healing, epidermal keratinocytes play essential roles in the secretion of factors that promote angiogenesis. However, specific cues in the wound microenvironment that trigger the production of pro-angiogenic factors by keratinocytes, and the cellular receptors that mediate this response, remain unclear. In this study, we exploited a model of conditional integrin knockout to demonstrate impaired wound angiogenesis in mice that lack α3β1 integrin in epidermis. These findings identify a novel role for α3β1 integrin in promoting wound angiogenesis through a mechanism of crosstalk from epidermal to endothelial cells, and they implicate MRP3 in this integrin-dependent crosstalk. Such a mechanism represents a novel paradigm for integrin-mediated regulation of wound angiogenesis that extends beyond traditional roles for integrins in cell adhesion and migration.

Key words: Integrin, MRP3 (Prl2c4), Proliferin, Keratinocyte, Wound healing

Introduction
Cutaneous wound healing is a complex process requiring collaborative interactions between distinct cell types that reside in different compartments of the skin, including epidermal keratinocytes, dermal fibroblasts, macrophages, neutrophils and vascular endothelial cells (Martin, 1997; Singer and Clark, 1999). Following wounding, the rapid regeneration of the epidermis, or re-epithelialization, is required to cover the denuded dermal surface of the wound and restore the barrier function and integrity of the skin. Keratinocyte migration and proliferation are crucial for this process (Santoro and Gaudino, 2005). In addition to their roles in re-epithelialization, wound keratinocytes secrete cytokines, growth factors and extracellular proteases that promote angiogenesis (Maldener et al., 1998; Salo et al., 1994; Santoro and Gaudino, 2005; Singer and Clark, 1999), thereby mediating crosstalk from keratinocytes to endothelial cells that promotes wound healing. Activation of wound-edge keratinocytes is probably triggered by newly encountered elements of the wound environment, including serum factors and exposed or newly synthesized cell-adhesion ligands in the provisional extracellular matrix (ECM) (Grinnell, 1992; Martin, 1997; Singer and Clark, 1999). However, specific extracellular cues in the wound environment that promote production of pro-angiogenic factors by keratinocytes remain unknown.

The mitogen-regulated proteins/proliferins (MRPs) are members of the prolactin growth hormone superfamily that comprise a group of highly homologous, growth-factor-inducible genes with roles in promoting endothelial cell proliferation, migration and angiogenesis during fetal and placental development (Corbacho et al., 2002; Fassett and Nilsen-Hamilton, 2001; Jackson et al., 1994; Soares et al., 2007; Toft et al., 2001). MRPs regulate endothelial cell migration and proliferation through interactions with cell-surface receptors, including the insulin-like growth factor 2 (IGF2)/mannose 6-phosphate receptor, MRP/proliferin receptor, or other unidentified receptors (Corbacho et al., 2002; Jackson et al., 1994; Nelson et al., 1995; Volpert et al., 1996). For example, endothelial cell migration can be induced by MRP binding to the IGF2 receptor and activation of a G-protein-coupled, mitogen-activated protein kinase (MAPK) pathway (Groskopf et al., 1997). Some studies have shown that increased MRP gene expression is associated with cellular immortalization and transformation (Malyankar et al., 1994; Parfett et al., 1985). Another study identified a novel role for MRP3, also known as PRL2C4 (prolactin family 2, subfamily c, member 4), as a likely regulator of angiogenesis during cutaneous wound healing (Fassett and Nilsen-Hamilton, 2001). Indeed, MRP3 gene expression is induced in activated wound keratinocytes, and MRP3 protein continues to accumulate with peak expression occurring 4-5 days post-injury, corresponding well with the timing...
of wound angiogenesis and suggesting that MRP3 secretion by keratinocytes may promote angiogenesis during wound repair (Fassett and Nilsen-Hamilton, 2001). MRP3 expression can be induced in cultured keratinocytes by keratinocyte growth factor (KGF) (Fassett and Nilsen-Hamilton, 2001). However, extracellular cues that induce MRP3 secretion by keratinocytes during in vivo wound healing are not yet known.

A key to understanding the pathogenesis of chronic wounds, and age-related wound defects, is identifying cellular receptors that mediate responses of the epidermis to provisional wound matrix, and determining how changes in these receptors contribute to impaired wound healing. Integrins are the major cell-surface receptors for cell adhesion and migration (Hynes, 2002), and epidermal keratinocytes express several integrins that bind ECM ligands in provisional wound ECM (Litjens et al., 2006; Watt, 2002). Although epidermis-specific deletion of the β1 integrin subunit have caused severe wound healing defects (Grose et al., 2002), knockouts of individual integrins have had surprisingly mild effects on wound healing, and in vivo roles for some keratinocyte integrins remain unclear (Grenache et al., 2007; Litjens et al., 2006; Zweers et al., 2007). α3β1 integrin is expressed in both resting and wounded epidermis, where its main ECM ligand is laminin-332/laminin-5 (LN-332; previously known as laminin-5, kalinin, nicein or epiligrin). Newborn mice that lack α3β1 integrin owing to the Iγga3 (which encodes α3 integrin-null mutation display epidermal microblister and organizational defects in the cutaneous basement membrane (DiPersio et al., 1997), which persist to some extent into adulthood of conditional Iγga3-knockout mice lacking α3β1 integrin in epidermis (Margadant et al., 2009). In addition, studies in both mouse and human keratinocytes have supported an important role for α3β1 integrin and LN-332 in the regulation of polarized cell spreading and migration (Choma et al., 2004; Frank and Carter, 2004; Hamelers et al., 2005; Nguyen et al., 2000). Whereas keratinocyte migration is clearly facilitated by other integrins that also bind ligands present in the wound ECM, such as fibronectin and exposed dermal collagen (Clark et al., 1982; Nguyen et al., 2000; Pilcher et al., 1997), keratinocytes that are deficient in LN-332 show defects in migration even on collagen (Ryan et al., 1999), suggesting an important role for LN-332 deposition during epidermal migration over the provisional ECM of the wounded bed. Consistently, de novo synthesis of precursor LN-332 is an early event in the activation of wound-edge keratinocytes (Litjens et al., 2006; Nguyen et al., 2000). However, two recent in vivo studies performed in distinct genetic models have indicated either pro-migratory or anti-migratory effects of α3β1 integrin during wound re-epithelialization (Margadant et al., 2009; Reynolds et al., 2008), suggesting that roles for this integrin in wound closure are more complex than previously anticipated (see Discussion).

In contrast to their widely studied roles in keratinocyte migration, proliferation and matrix assembly, potential roles for epidermal integrins in regulating the production of pro-angiogenic factors that promote wound angiogenesis have not been extensively explored. In the present study we have exploited a murine model of α3-integrin knockout to assess the effects of α3β1-integrin deficiency in epidermal keratinocytes on wound angiogenesis in vivo and endothelial cell function in vitro. We demonstrate that ablation of α3β1 integrin in the epidermis by conditional Iγga3 knockout results in impaired wound angiogenesis in vivo. In addition, we use a combination of genetic and shRNA approaches to show that α3β1 integrin induces MRP3 mRNA expression and protein secretion in cultured keratinocytes, and that this regulation is required for keratinocyte-to-endothelial-cell crosstalk, which promotes endothelial-cell migration. Finally, we show that impaired wound angiogenesis in epidermis-specific α3-integrin-knockout mice is correlated with reduced expression of MRP3 in the wounded epidermis. These findings reveal a novel role for α3β1 integrin in promoting keratinocyte-to-endothelial-cell crosstalk by regulating expression in keratinocytes of a pro-angiogenic factor, MRP3, and they provide the initial evidence that this regulation promotes wound angiogenesis in vivo. Our findings may therefore represent a novel paradigm for the regulation of wound healing by certain integrins expressed in epidermis that extends beyond their traditional, well-documented roles in regulating cell adhesion and migration.

Results

Derivation of mice with epidermis-specific deletion of α3β1 integrin

To test the importance of α3β1 integrin expression in the epidermis for wound angiogenesis, we exploited conditional knockout mice with epidermis-specific deletion of the α3-integrin subunit (summarized in Fig. 1A). Mice with floxed Iγga3 alleles (referred to here as Iγga3flx/flx mice), in which exon 3 was flanked by loxP sites, were crossed with Iγga3flx/+ mice that express Cre recombinase under control of the keratin 14 promoter (K14-Cre), which restricts Cre expression to basal keratinocytes of the interfollicular and follicular epidermis, including the stem-cell compartment in the hair follicle (Castilho et al., 2007; Huelskens et al., 2001). The resulting mice of the K14-Cre:Iγga3floxflox genotype were then backcrossed to obtain K14-Cre:Iγga3floxflox mice. Where indicated, some experiments utilized mice of the K14-Cre:Iγga3floxflox genotype, in which one of the Iγga3 alleles corresponds to the conventional Iγga3-null allele described previously (Kreidberg et al., 1996). Hereafter, K14-Cre:Iγga3floxflox mice or K14-Cre:Iγga3floxflox mice are referred to as α3eKO mice; control mice were littermates that lacked K14-Cre and were referred to as Iγga3floxflox or Iγga3floxflox genotype, as appropriate.

Immunofluorescence of frozen skin sections from control mice that were of the Iγga3floxflox genotype, but lacked the K14-Cre transgene, showed that α3-integrin staining was easily detected in basal keratinocytes of the epidermis (Fig. 1F), as expected (DiPersio et al., 1997). By contrast, specific α3-integrin staining was absent from the epidermis of K14-Cre:Iγga3floxflox mice, confirming undetectable expression of α3β1 integrin in the epidermis of α3eKO mice (Fig. 1G; asterisks in Fig. 1F,G indicate non-specific staining in the cornified layer that was also seen with the corresponding pre-immune serum; not shown). Skin sections stained with Hematoxilin and Eosin (H&E) did not reveal gross differences in the morphology or thickness of the interfollicular epidermis between control and α3eKO mice (Fig. 1B,C), consistent with essentially normal development and stratification of interfollicular epidermis in previous studies of α3β1-integrin-deficient skin (Conti et al., 2003; DiPersio et al., 1997). However, we noticed a reduction in the size of hair follicles in α3eKO mice compared with control mice, as evident in the images of Fig. 1B,C. This observation is reminiscent of previously reported defects in hair follicle development in Iγga3-null skin grafts (Conti et al., 2003), although less severe than the progressive loss of hair follicles that was reported in adult mice with epidermis-specific deletion of the β1 integrin subunit (Brakebusch et al., 2000). A recent study using a similar model of epidermis-specific deletion of α3 integrin reported inflammation around the eyes and local hair loss (alopecia) that appeared around 3 to 4 months after birth and progressed with age (Margadant et al., 2009). We observed similar phenotypes in
α3eKO mice (not shown), although extensive alopecia was not usually obvious until several months later, possibly reflecting differences in genetic background.

LN-332 staining was detected in the cutaneous basement membrane of both control and α3eKO mice (Fig. 1H,I), consistent with previous reports that α3β1 integrin is not required for LN-332 synthesis in vivo (DiPersio et al., 1997; DiPersio et al., 2000b). Margadant and co-workers reported occasional microblistering at the dermal-epidermal junction in α3β1-integrin-deficient epidermis of adult (1-year-old) mice (Margadant et al., 2009), a phenotype reminiscent of the microblistering that accompanies extensive basement membrane defects in Itga3-null neonatal mice (DiPersio et al., 1997). Although we did not observe such defects in the skin of young adult mice (~4 months) used in our current study, it seems likely that more extensive analysis over a greater age range may reveal occasional basement membrane defects and microblistering.

The reduced incidence and severity of these phenotypes in mature α3eKO mice may indicate partial recovery in adult mice from basement membrane defects that are more prominent in Itga3-null neonatal mice (DiPersio et al., 1997). Alternatively, it is possible that the more extensive basement membrane defects seen in newborn Itga3-null mice reflect a requirement for α3β1 integrin in other skin cell types from which α3 integrin is also absent, or at a stage of prenatal development that precedes efficient K14-Cre-mediated deletion of the Itga3$^{floxed}$ allele (see Materials and Methods for details). Frt and LoxP sites floxed Itga3 expression is absent from epidermis of α3eKO mice. To assess α3-integrin expression in wounded epidermis, frozen sections were prepared 5 days post-injury from 4-mm punch biopsy wounds of α3-integrin control and α3eKO mice and stained by immunofluorescence for α3 integrin. In control mice, α3 integrin was easily detected in keratinocytes of the migrating epidermal tongue (Fig. 2E). By contrast, α3-integrin expression remained undetectable in the wound-edge keratinocytes of α3eKO mice (Fig. 2F), and staining was comparable to that seen with the corresponding pre-immune serum (Fig. 2G,H), confirming that there was no expansion of residual cells that retained detectable levels of α3β1 integrin.

Previous studies in cultured Itga3-null keratinocytes (deHart et al., 2003; DiPersio et al., 2000a) and epidermis of Itga3-null mice (DiPersio et al., 2000b) showed that α3β1 integrin is not required for LN-332 expression by epidermal keratinocytes. However, it has been suggested that α3β1-integrin-mediated signaling in keratinocytes may regulate the deposition of LN-332 during wound repair in vivo (Hamelers et al., 2005). Therefore, we also examined LN-332 staining at the wound edge. LN-332 was present around the migrating epidermis of both control and α3eKO mice, and its distribution reflected that it was not yet organized into basement membrane (Fig. 2I,J), indicating that α3eKO keratinocytes are capable of depositing LN-332 into the wound and that α3β1 integrin is not essential for LN-332 expression. However, we cannot rule out the possibility that proteolytic processing of LN-332, or its incorporation into newly synthesized basement membrane, is altered in α3eKO wounds.
α3β1 integrin regulates MRP3 and angiogenesis

Mice that lack α3β1 integrin in the epidermis display reduced angiogenesis during wound healing

Epidermal keratinocytes are well known to secrete pro-angiogenic factors during wound healing (Santoro and Gaudino, 2005; Singer and Clark, 1999), although a role for integrins in this regulation has not been extensively explored. To test if α3β1 integrin in the epidermis is important for induction of wound angiogenesis in vivo, frozen sections were prepared 5 days post-injury from control and α3eKO mice and stained with anti-CD31/PECAM 1 to label blood vessels. As expected, control mice (Itga3flx/flx; α3+ cells) showed increased blood vessel density in dermis directly adjacent to the wound bed, compared with dermis distal to the wound (Fig. 3A, control), indicative of robust induction of angiogenesis during this phase of wound repair. Blood vessel density in these mice was similar to that observed in wounded skin of Itga3+/+ wild-type mice (data not shown), indicating that one functional Itga3 allele was sufficient for normal wound angiogenesis. By contrast, wounds from α3eKO mice (K14-Cre::Itga3flx/flx) showed substantially reduced blood vessel density compared with wounds from control mice (Fig. 3A, α3eKO). Quantification of these data revealed that α3eKO mice displayed a statistically significant reduction in blood vessel density of about 60% compared with control mice (Fig. 3B). These results indicate that α3β1 integrin is required in epidermis for full induction of angiogenesis during wound healing.

α3β1 integrin in keratinocytes regulates the secretion of a pro-angiogenic factor(s) that promotes endothelial-cell migration

The impaired angiogenesis that we observed in wounds of α3eKO mice suggests a novel role for this integrin in promoting crosstalk between the epidermal and endothelial compartments of the skin. Therefore, we next tested if α3β1 integrin in epidermal keratinocytes is required for the crosstalk to endothelial cells that occurs through secretion of soluble factors that promote endothelial-cell migration. For these studies we utilized keratinocyte cell lines derived from a wild-type mouse (WT cells) or an Itga3-null mouse (α3– cells) (DiPersio et al., 2000a). As an additional control, α3β1-integrin expression was restored in the Itga3-null cell line by stable transfection with a cDNA encoding the human α3-integrin subunit (α3+ cells) (Iyer et al., 2005). Transwell assays were performed to measure relative migration of human umbilical vein endothelial cells (HUVECs) in response to factors secreted by keratinocytes. Serum-starved HUVECs were seeded into the upper chambers of gelatin-coated transwell filters, then conditioned media collected from 24 hour confluent cultures of WT, α3– or α3+ keratinocytes were added to the lower chambers and tested for effects on HUVEC migration over a 4-hour period. There was a significant stimulation of HUVEC migration in response to conditioned medium from WT keratinocytes, compared with basal migration seen in response to unconditioned medium. By contrast, HUVEC migration in conditioned medium from α3– keratinocytes was similar to basal levels (Fig. 4). Importantly, ability to stimulate HUVEC migration was restored in α3– cells that were rescued with human α3 integrin (Fig. 4). These data indicate that α3β1-integrin deficiency in keratinocytes leads to reduced secretion of factors that induce endothelial-cell migration.

α3β1 integrin is required in keratinocytes for expression of the pro-angiogenic factor MRP3

We next wanted to identify the α3β1-integrin-dependent factor(s) involved in keratinocyte-mediated induction of endothelial-cell migration. Reduced wound angiogenesis observed in α3eKO mice (Fig. 3) prompted us to ask whether α3β1 integrin is required in keratinocytes for expression of known pro-angiogenic factors in wound healing. We focused our initial attention on VEGF, MMP9 and MRP3, all of which are expressed in keratinocytes and have been previously implicated in wound angiogenesis (Fassett and Nilsen-Hamilton, 2001; Madlener et al., 1998; Salo et al., 1994; Santoro and Gaudino, 2005; Singer and Clark, 1999). First, total RNA was isolated from α3+/+ and α3– keratinocytes and analyzed for expression of these pro-angiogenic factors. Interestingly, Mrp3 mRNA was considerably more abundant in α3+ cells than in α3– cells (Fig. 5A). Mmp9 mRNA expression was also higher in α3+ cells than in α3– cells, which we have shown previously (Iyer et al., 2005), and served as a positive control for α3β1-integrin-
dependent effects in this assay (Fig. 5A). We did not detect α3β1-integrin-dependent effects on expression of Vegf mRNA (Fig. 5A). In accordance with α3β1-integrin-dependent effects on Mrp3 mRNA, MRp3 protein was easily detected in cell lysates from both α3+ and WT keratinocytes, but it was barely detectable in lysates from α3– keratinocytes (Fig. 5B). An upper non-specific band, which was also detected by the corresponding pre-immune serum (not shown), served as an additional loading control (Fig. 5B, asterisk). Quantification of data from four independent experiments indicated that total MRp3 protein, after normalization to keratin 14, was decreased to approximately 5% in α3β1-integrin-deficient cells compared with either of the α3β1-integrin-expressing cell lines (Fig. 5C). Assessment of conditioned culture medium confirmed that α3β1-integrin-expressing keratinocytes secreted much higher levels of MRp3 than α3-integrin-deficient keratinocytes (Fig. 5B). These data indicate that α3β1 integrin promotes MRp3 expression and secretion in vitro, and they identify this pro-angiogenic factor as a candidate for mediating α3β1-integrin-dependent crosstalk from keratinocytes to endothelial cells that promotes wound angiogenesis.

α3β1-integrin-dependent expression of MRp3 in keratinocytes stimulates endothelial-cell migration

To test whether α3β1-integrin-dependent expression and secretion of MRp3 (Fig. 5) is involved in the keratinocyte-mediated induction of endothelial-cell migration (Fig. 4), α3β1-integrin-expressing keratinocytes were stably infected with lentiviruses that express shRNAs that target murine Mrp3, or non-targeting shRNA as a control. Mrp3 mRNA was decreased by 70% or more in cells expressing each of three independent shRNAs that target this transcript, compared with cells infected with control shRNA, whereas β-actin mRNA levels were unaffected by either control or targeting shRNAs (Fig. 6A). Immunoblot analysis of conditioned medium from these cells confirmed efficient suppression of secreted MRp3 protein levels by each Mrp3 shRNA (Fig. 6B). The HUVEC migratory response to conditioned medium from keratinocytes that express control shRNA was about 2.5-fold higher than the basal response to unconditioned medium. By contrast, the migratory response to conditioned medium from cells that express Mrp3-targeting shRNA was reduced to basal levels (Fig. 6C). Importantly, similar results with three different shRNAs that target distinct regions of the murine Mrp3 transcript indicate that this inhibition was not due to off-target RNAi effects. Poor transfection efficiency of the α3– keratinocytes precluded our ability to restore MRp3 expression to endogenous levels seen in WT cells. However, we found that conditioned medium from 293 cells that overexpress recombinant, His-tagged MRp3 at high levels induced HUVEC migration only 1.2-fold above levels seen in response to conditioned medium from control 293 cells (@P<0.046, t-test; data not shown). Although we cannot rule out cell-specific modifications of MRp3 or inhibitory effects of the His-tag, this result may indicate that other keratinocyte factors in addition to MRp3 are required to induce a robust migratory response. Nevertheless, results in Fig. 6 show that α3β1-integrin-dependent MRp3 secretion in keratinocytes plays a crucial role in mediating crosstalk to endothelial cells that promotes their migration. These findings support a model wherein
α3β1-integrin-mediated expression of MRP3 in the epidermis contributes to wound angiogenesis in vivo.

Depletion of α3β1 integrin from epidermis leads to reduced MRP3 expression during wound healing in vivo

MRP3 is the major MRP gene expressed in the epidermis of wounds, and it has been identified as a likely pro-angiogenic factor during wound healing (Fassett and Nilsen-Hamilton, 2001). MRP3 gene expression is induced soon after wounding, and the MRP3 protein continues to accumulate in the epidermis to peak levels 4-5 days post-injury, consistent with a role in promoting wound angiogenesis (Fassett and Nilsen-Hamilton, 2001). To test if α3β1 integrin regulates MRP3 expression in wounded epidermis, frozen skin sections from control or α3eKO mice were immunostained with anti-MRP3 antibody. MRP3 levels were low or absent in normal, unwounded skin of both control and α3eKO mice (data not shown). Immunostaining of epidermis adjacent to 5-day wounds indicated that MRP3 expression was detected in the majority of control wounds (7/8 wounds examined), within both wound-proximal hair follicles (Fig. 7A, lower panels) and the migrating epidermal tongue (Fig. 7B, upper panels), consistent with previous findings that MRP3 is induced in hair follicles near the wound and in wound-edge epidermis by 5 days post-injury (Fassett and Nilsen-Hamilton, 2001). Interestingly, specific MRP3 staining was markedly reduced or absent in wound-proximal hair follicles (Fig. 7A, lower panels) or wound-edge epidermis (Fig. 7B, lower panels) in the majority of α3eKO wounds (8/11 wounds examined). The lack of a complete correlation in α3eKO mice between absence of α3β1 integrin and reduced MRP3 is likely to reflect partial compensation by other factors present in the complex wound microenvironment that can also induce MRP3, such as KGF (Fassett and Nilsen-Hamilton, 2001). Nevertheless, our observations that α3β1-integrin deficiency leads to both reduced MRP3 expression in wounds (Fig. 7) and reduced wound angiogenesis (Fig. 3) support an in vivo role for α3β1-integrin-mediated induction of MRP3 during wound angiogenesis.

Discussion

Although an extensive literature supports important roles for integrins in angiogenesis, most previous studies have focused on integrins expressed on endothelial cells that function within these cells to regulate essential processes such as differentiation, proliferation, migration and survival (for reviews, see Hynes, 2007; Silva et al., 2008). By contrast, results from the current study identify a distinct and novel role for α3β1 integrin in regulating crosstalk from the epidermis to the vasculature of the skin to promote wound angiogenesis (depicted in Fig. 8). Indeed, α3β1-integrin-mediated crosstalk between distinct tissue compartments of the skin may represent a novel paradigm of integrin control over angiogenesis that extends beyond their well-established roles in the regulation of cell adhesion and migration. We observed that wounds in mice with an epidermis-specific knockout of the α3-integrin subunit displayed markedly reduced blood-vessel density compared with wounds from control mice. Additionally, endothelial-cell migration in vitro was reduced in response to conditioned medium from α3β1-integrin-deficient keratinocytes, compared with α3β1-integrin-
expressing keratinocytes, indicating that α3β1 integrin is required for keratinocyte-to-endothelial-cell crosstalk. Finally, our data demonstrate a novel function for α3β1 integrin in the induction of MRP3 gene expression in keratinocytes that promotes endothelial-cell migration, and they support a role for this regulation during wound angiogenesis in vivo.

Several different integrins have been shown to be important for normal skin development or post-developmental skin functions such as wound healing (Litjens et al., 2006; Watt, 2002). Recent studies have shown that mice deficient for all β1 integrins in the epidermis have severe defects in wound re-epithelialization and hair-follicle morphogenesis (Brakebusch et al., 2000; Grose et al., 2002; Raghavan et al., 2000). One of the major α-subunit-binding partners for β1 integrins in the epidermis is the α3 subunit, and α3β1 integrin has important roles in maintaining basement membrane integrity in vivo and keratinocyte motility and survival in vitro (Choma et al., 2004; DiPersio et al., 1997; Frank and Carter, 2004; Manohar et al., 2004). However, two recent studies performed in distinct genetic models have reported either pro-migratory or anti-migratory effects of this integrin on wound re-epithelialization in vivo. Indeed, assessment of wound healing in full-thickness skin grafts from Itga3-null neonatal mice showed that absence of α3β1 integrin from skin results in reduced re-epithelialization, suggesting a pro-migratory role (Reynolds et al., 2008). By contrast, epidermis-specific deletion of α3 integrin caused slightly enhanced wound re-epithelialization, indicating that α3β1 integrin delays, rather than facilitates, wound closure in this model (Margadant et al., 2009). Although we did not perform an extensive analysis of wound re-epithelialization in the current study, our preliminary observations revealed no obvious reduction in wound re-epithelialization of α3eKO mice, consistent with the findings of Sonnenberg and coworkers (Margadant et al., 2009). It remains to be determined whether the disparate findings in the above-mentioned studies stem from inherent differences in the in vivo models that were used (i.e. total versus epidermis-specific α3-integrin knockout), or from different requirements for α3β1 integrin in neonatal skin (Reynolds et al., 2008) versus adult skin (Margadant et al., 2009). Nevertheless, the combined observations that α3eKO mice show reduced wound angiogenesis (current study), and that epidermis-specific deletion of α3β1 integrin does not impair (and appears to enhance) wound re-epithelialization (Margadant et al., 2009), suggest that α3β1-integrin-mediated wound angiogenesis is not crucial for the re-epithelialization step of wound healing in this model. This observation is consistent with a previous report that inhibiting angiogenesis did not affect the rate of re-epithelialization of full-thickness excisional wounds in mice (Michaels et al., 2005). It will be interesting in future studies to test if α3β1-integrin deficiency in epidermis alters other aspects of the wound-healing process that have been linked to angiogenesis, such as formation of granulation tissue or wound tensile strength.

As keratinocytes migrate across the wound bed, they secrete multiple cytokines and growth factors that facilitate the healing process, including pro-angiogenic factors that can act upon endothelial cells to induce angiogenesis (Santoro and Gaudino, 2005; Singer and Clark, 1999). Our data identify MRP3 as a potentially important α3β1-integrin-dependent factor secreted by keratinocytes to promote angiogenesis. Consistently, MRPs are known to regulate angiogenesis during development (Corbacho et al., 2002; Jackson et al., 1994), and MRP3 expression in wound keratinocytes and hair follicles adjacent to the wound is thought to
promote wound angiogenesis (Fassett and Nilsen-Hamilton, 2001). Our combined findings that MRP3 expression was α3β1-integrin dependent in keratinocytes, and that shRNA-mediated suppression of MRP3 in α3β1-integrin-expressing keratinocytes eliminated the ability to stimulate endothelial-cell migration, identify MRP3 as a mediator of α3β1-integrin-dependent keratinocyte-to-endothelial-cell crosstalk. Interestingly, shRNA-mediated suppression of MMP9, another α3β1-integrin-dependent pro-angiogenic factor (Iyer et al., 2005), did not consistently alter endothelial-cell migration in response to keratinocyte-conditioned medium (data not shown), suggesting that MMP9 is not required for this response, at least in vitro. Importantly, our in vivo model revealed a correlation between reduced MRP3 expression and absence of α3β1 integrin in wound-proximal hair follicles and wound-edge keratinocytes of α3eKO mice. These findings indicate that α3β1 integrin regulates MRP3 expression in vivo and support a role for MRP3 in the α3β1-integrin-dependent wound angiogenesis that we observed in these mice. It is well known that hair follicles include a reservoir of stem cells that may contribute to wound re-epithelialization, as cells from the hair follicle bulge migrate outward to repair the damaged epidermis (Ito et al., 2005; Levy et al., 2005). It is possible that activated keratinocytes in wound-proximal hair follicles secrete MRP3, which then diffuses into the adjacent wound and acts upon endothelial cells at the site of injury to promote their migration and drive angiogenesis.

To the best of our knowledge, the current study is the first to show that MRP3 can be regulated by integrins, and it remains to be determined whether the ability to induce MRP3 expression is specific to α3β1 integrin or also extends to other integrins. Importantly, expression of other integrins is not altered in the α3β1-integrin-deficient keratinocytes used in this study (DiPersio et al., 2000a). In addition, several studies have indicated that in vivo deletion of α3 integrin does not alter other integrins in the epidermis (DiPersio et al., 2000b; Hodivala-Dilke et al., 1998; Margadant et al., 2009). Based on these previous studies, together with our current findings that in the absence of α3β1 integrin other endogenous keratinocyte integrins do not support robust wound angiogenesis in vivo (Fig. 3), stimulation of endothelial-cell migration in vitro (Fig. 4) or MRP3 expression (Fig. 5), we think compensation by other integrins is an unlikely explanation for the MRP3 expression that we observed in a small proportion of α3β1-integrin-deficient wounds. Instead, we suggest that residual MRP3 expression may result from soluble factors present in the wound microenvironment that can also induce MRP3 in keratinocytes, such as KGF (Fassett and Nilsen-Hamilton, 2001). Thus, it seems likely that α3β1 integrin and certain growth factors are both required to achieve consistent and efficient induction of MRP3 during wound healing.

In addition to functioning as an adhesion receptor for laminins in the ECM, there is evidence that α3β1 integrin can function as a signaling component from within cell-cell junctional complexes that include E-cadherin (also known as cadherin 1) and the tetraspanin CD151 (Chattopadhyay et al., 2003). Importantly, α3β1-integrin expression in quiescent epidermis (where it is localized largely to cell-cell junctions) does not induce MRP3 expression or an angiogenic response, whereas our results show that α3β1 integrin promotes these responses in wounded epidermis. These observations suggest that wound-associated changes in α3β1 integrin, such as ligation of newly synthesized LN-332 (Nguyen et al., 2000), may be required for induction of MRP3. Direct testing of a requirement for LN-332 is complicated by the fact that cultured keratinocytes rapidly deposit abundant LN-332 onto their ECM substrate (Nguyen et al., 2000). However, future studies using laminin-binding mutants of α3β1 integrin should help determine whether ligation to LN-332 is required for MRP3 induction. Interestingly, LN-332 expression is also elevated in invasive squamous cell carcinomas (Marinkovich, 2007), and recent studies in our lab indicate that α3β1 integrin promotes subcutaneous tumor growth of oncogenically transformed keratinocytes (Lamar et al., 2008). Given the role for α3β1 integrin in wound angiogenesis that we have identified in the current study, it is possible that this integrin plays a similar role in mediating crosstalk between carcinoma cells and endothelial cells to promote tumor angiogenesis. Indeed, previous studies have implicated MRP3s in promoting tumor angiogenesis (Bengtson and Linzer, 2000; Corbacho et al., 2002; Toft et al., 2001).

In summary, this study identifies a novel role for α3β1 integrin in the epidermis in promoting wound angiogenesis, and it identifies α3β1-integrin-mediated induction of MRP3 expression as a novel mechanism of keratinocyte-to-endothelial-cell crosstalk. As these findings extend the roles of epidermal integrins beyond their traditional roles in regulating cell adhesion and migration, they further our understanding of how integrins mediate epidermal responses to provisional wound ECM that contribute to normal wound healing. Integrins are particularly attractive targets for therapeutic agents aimed at enhancing wound healing, as they are accessible on the cell surface and can be manipulated with relative ease. The novel pro-angiogenic function of α3β1 integrin described here, together with previously studied functions of α3β1 integrin in keratinocyte migration, basement membrane assembly and wound re-epithelialization (Choma et al., 2004; delHart et al., 2003; Frank and Carter, 2004; Hamelers et al., 2005; Margadant et al., 2009; Reynolds et al., 2008), suggest multiple and complex roles for this integrin that may be simultaneously exploitable in therapeutic strategies to facilitate wound repair.

Materials and Methods
Mice
Generation of mice that carry the conventional Itga3-null mutation was described previously (Kreidberg et al., 1996). The targeting vector to generate the floxed Itga3
Transwell assays of endothelial-cell migration

Transwell migration assays were performed as described (Meadows et al., 2004). Briefly, transwell tissue-culture inserts (Costar, Corning, NY) with 8 μm pores were coated with 0.2% gelatin. HUVECs were serum-starved for 24 hours, trypsinized and seeded onto top surfaces in serum-free MCD-131 at 5 × 10^4 cells per insert. Lower chambers contained serum-free MCD-131 medium that had been conditioned by wild-type, α3+ or α3− keratinocytes, in a 70:30 ratio with complete EGM-2 medium (Lonza, Walkersville, MD). Unconditioned medium in the same 70:30 ratio was used to establish baseline cell migration. For some experiments, conditioned medium was collected from 293 cells that overexpress recombinant His-tagged MR3P, and from untransfected 293 cells as a control. After 4 hours of migration, cells were fixed in 3.7% formaldehyde/PBS and stained with 0.5% Crystal Violet. Non-migratory cells on the top surface were removed with cotton swabs and cells that had migrated to the bottom surface were permeabilized with 0.1% Triton X-100 and stained with DAPI (Pierce). Cells were visualized using an Olympus inverted IX70 microscope, and three random 10× fields were collected using a SensCam digital camera (Cooke, Eugene, OR). The total cell number per field was quantified using ImagePro Plus (Media Cybernetics, Silver Spring, MD).

RT-PCR

Total RNA was isolated from mouse keratinocytes using the Purescript RNA Isolation Kit (Genta Systems, Minneapolis, MN) or Trizol Reagent (Invitrogen Corporation), then reverse transcribed to produce cDNA using the First-Strand cDNA Synthesis kit (Promega). PCR reactions were carried out in PCR REDTaq ready mix (Sigma). PCR primers and conditions for amplification of Mmp9 were previously described (Iyer et al., 2005). PCR primers and conditions for amplification of Mrp3: forward primer, 5'-CCTCCTCTCTGATCTACAAGGAT-3', reverse primer 5'-CATGTAACACTACACCACGACGCA-3' (forward) and 5'-ACTCTAAGGCCCATATCTCCTC-3' (reverse) and the following reaction conditions: denaturation at 94°C for 30 seconds; extension at 58°C for 60 seconds; annealing at 72°C for 120 seconds; 30 amplification cycles. PCR generates a 542 bp product for the wild-type Itga3 allele, and a 623 bp product for the Itga3 allele. PCR genotyping for the K14-Cre transgene was performed using the primers 5'-ATGTCACAGTTCCG-3' (forward) and 5'-CGCCGCCATAACACCGTAGTA-3' (reverse) and the following reaction conditions: denaturation at 94°C for 30 seconds; extension at 49°C for 60 seconds; annealing at 72°C for 90 seconds; 32 amplification cycles. PCR generates a 370 bp product for the Cre transgene. Cre-mediated excision of exon 3 from the Itga3 allele results in translational termination within the coding sequence of the upstream α3-integrin. The primer pair 5'-CGCCTAGAAGC-3' and 5'-CATGTAACACTACACCACGACGCA-3' was used to establish baseline cell migration. For some experiments, conditioned medium was collected from 293 cells that overexpress recombinant His-tagged MR3P, and from untransfected 293 cells as a control. After 4 hours of migration, cells were fixed in 3.7% formaldehyde/PBS and stained with 0.5% Crystal Violet. Non-migratory cells on the top surface were removed with cotton swabs and cells that had migrated to the bottom surface were permeabilized with 0.1% Triton X-100 and stained with DAPI (Pierce). Cells were visualized using an Olympus inverted IX70 microscope, and three random 10× fields were collected using a SensCam digital camera (Cooke, Eugene, OR). The total cell number per field was quantified using ImagePro Plus (Media Cybernetics, Silver Spring, MD).

Western blot

Keratinocytes were cultured on collagen-coated dishes in the absence of serum for 24 hours. Conditioned medium was collected and cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce). Samples of equal protein content, or equivalent proportions of culture supernatant, were assayed by immunoblot using rabbit anti-MRP3 or the corresponding pre-immune serum (1:500) (Fassett and Nilsen-Hamilton, 2001), or rabbit anti-keratin (1:1000) (Covance Inc, Princeton, NJ), followed by peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000) (Cell Signaling Technology, Beverly, MA). Chemiluminescence was performed with the SuperSignal kit (Pierce), and signals were visualized using a Bio-Rad FluorS 2000 and quantified using Quantity One software (Bio-Rad, Hercules, CA).

shRNA-mediated suppression of MR3P

For use of shRNA to stably suppress MR3P, we used MISSION™ lentiviral shRNA constructs (Sigma) encoding shRNAs that target murine Mrp3, or a non-targeting control shRNA. Lentiviral constructs were transfected into the packaging cell line, 293FT using lipofectamine plus reagent (Invitrogen Corporation). Viral supernatants were added to α3-integrin-expressing keratinocytes for 24 to 48 hours, followed by culture in keratinocyte growth medium containing 10 μM puromycin (MP Biomedicals, Solon, OH) to select populations of stably transduced cells. All experiments and reagents described in this study were approved by the Institutional Biosafety Committee of Albany Medical College. All animal studies were approved by the Institutional Animal Care and Use Committee of Albany Medical College.

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