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Opposing roles of epidermal integrins α3β1 and α9β1 in regulation of mTLD/BMP-1-mediated laminin-γ2 processing during wound healing

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Abstract

Proteolytic processing of the laminin-γ2 chain is a hallmark of basement membrane maturation in the skin. Integrin α3β1, a major receptor for epidermal adhesion to laminin-332, is critical for proper basement membrane organization during skin development and wound healing. Previously, we identified a role for α3β1 in promoting the processing of laminin-γ2 in cultured keratinocytes *in vitro* and in wound epidermis *in vivo*. In the current study we identify the *Bmp1* gene, which encodes variants of the mammalian tolloid/bone morphogenetic protein-1 metalloproteases, as a critical regulator of α3β1-dependent laminin-γ2 processing, thereby expanding the role of this integrin in controlling the secretion by the epidermis of factors that modulate the tissue microenvironment. Since our previous studies identified another epidermal integrin, α9β1, as a suppressive regulator of α3β1-dependent wound angiogenesis, we investigated whether α9β1 has a similar cross-suppressive effect on ability of α3β1 to promote basement membrane organization. Here, we demonstrate that, rather than a cross-suppressive role, α9β1 has an opposing role in basement membrane assembly/maturation through reduced laminin-γ2 processing via mammalian tolloid/bone morphogenetic protein-1. Indeed, while α3β1 promotes this process during wound healing, α9β1 has an inhibitory role, suggesting that regulation of basement membrane assembly requires a complex interplay between these distinct epidermal integrins.

INTRODUCTION

The cutaneous basement membrane (BM) is a specialized extracellular matrix (ECM) that acts as a physical barrier between the epidermis and dermis of the skin. Additionally, the

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CONFLICT OF INTEREST

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BM provides signals for intracellular pathways that modulate a range of keratinocyte functions including migration, survival, differentiation, and polarization. Keratinocyte-mediated changes to the BM, which may occur through ECM protein deposition or matrix proteolysis, can regulate many of the abovementioned keratinocyte functions.

Laminin-332 (LN-332), a major constituent of the epidermal BM, is made up of three chains designated α3, β3, and γ2 (Aumailley *et al.*, 2005; Aumailley *et al.*, 2003). In the mature BM of adult skin, proteolytic processing results in LN-332 which lacks the N-terminus of the laminin-γ2 (LNγ2) chain as well as the C-terminus of the α3 chain (Amano *et al.*, 2000; Marinkovich *et al.*, 1992; Sasaki *et al.*, 2001; Tsubota *et al.*, 2000). These processing events appear to modulate interactions of LN-332 with distinct ECM components, thereby regulating BM architecture (Aumailley *et al.*, 2003). For example, the L4 module within the N-terminus of unprocessed LNγ2 is required for stable incorporation of LN-332 into nascent BM (Gagnoux-Palacios *et al.*, 2001). The L4 module, notably, has binding sites for ECM components, such as nidogen-1 and fibulins-1 and -2, most of which are lost upon processing of the LNγ2 chain (Sasaki *et al.*, 2001). This suggests that some preliminary ECM linkages necessary for stable LN-332 incorporation during BM assembly are dispensable in mature BM.

Integrins are transmembrane proteins consisting of an α and a β subunit, and function as the main receptors for cell adhesion to the ECM (Hynes, 1992). Abundant in basal keratinocytes of the epidermis, integrins α6β4 and α3β1 mediate adhesion to the underlying BM (Janes and Watt, 2006; Longmate and DiPersio, 2014; Margadant *et al.*, 2010). LN-332 is the major adhesive ligand in the cutaneous BM for both α3β1 and α6β4 (Delwel *et al.*, 1994; Nguyen *et al.*, 2000), and inherited mutations in each of the individual LN-332 chains, as well as in the α3, α6 and β4 integrin subunits, lead to junctional forms of the human blistering skin disease, Epidermolysis Bullosa [reviewed in (Dang *et al.*, 2008; McGrath, 2015; Pulkkinen and Uitto, 1999)]. In addition to their well known roles in cell adhesion, several epidermal integrins have been shown to regulate ECM proteolysis and assembly through their abilities to control the expression or functions of MMPs or other ECM-degrading extracellular proteases, and changes in these integrin functions are likely to contribute to the pathologies of chronic wounds, blistering skin diseases and certain skin cancers [reviewed in (Longmate and DiPersio, 2014)]. Indeed, we recently identified α3β1 as an important mediator of LNγ2 processing, both *in vivo* during wound healing and in cultured keratinocytes under high calcium conditions that promote differentiated function (Longmate *et al.*, 2014). However, the extracellular protease(s) responsible for α3β1-dependent processing of LN-332 have not been identified previously.

While roles for distinct epidermal integrins during wound healing have been extensively investigated, understanding of how different integrins function cooperatively in keratinocytes to regulate wound healing has not been explored sufficiently (Longmate and DiPersio, 2014; Margadant *et al.*, 2010). Our previous work has established a major role for α3β1 in epidermal keratinocytes in promoting the secretion of proteases and growth factors that modulate the tissue microenvironment in skin (DiPersio *et al.*, 2000; Longmate *et al.*, 2014; Missan *et al.*, 2014; Mitchell *et al.*, 2009). Recently, we identified epidermal α9β1 as a cross-suppressive regulator of α3β1-mediated crosstalk from keratinocytes to endothelial

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cells that promotes wound angiogenesis (Longmate *et al.*, 2017). However, a cross-suppressive role for α9β1 in α3β1-mediated BM assembly has not been investigated previously. Here, we used mice with epidermis-specific deletion of α3β1, α9β1, or both α3β1 and α9β1, along with cultured mouse keratinocytes (MK cells) that express α3β1 or α9β1, separately or together, to determine how these two integrins coordinately regulate LN-332 processing and BM maturation. We demonstrate that α3β1 promotes LNγ2 processing at least in part through the upregulation of the *Bmp1* gene, which encodes variants of the mammalian tolloid/bone morphogenetic protein-1 (mTLD/BMP-1) metalloproteases that have been shown previously to cleave LNγ2 in cultured keratinocytes and in skin (Amano *et al.*, 2000; Muir *et al.*, 2016; Veitch *et al.*, 2003). Moreover, we show that α9β1 antagonizes both α3β1-dependent mTLD/BMP-1 expression and BM assembly/maturation. These findings indicate a complex interplay between these two integrins whereby α3β1 promotes, and coordinately, α9β1 inhibits mTLD/BMP-1 production and LNγ2 processing to effect timely BM assembly and maturation during wound healing.

RESULTS

Integrin α3β1 promotes mTLD/BMP-1-mediated LNγ2 processing in mouse keratinocytes

Previously, we reported that α3β1 is required for processing of the LNγ2 chain in cultured keratinocytes, as well as in the wound epidermis *in vivo* (Longmate *et al.*, 2014). Since mTLD/BMP-1 proteases are known to process LNγ2 (Amano *et al.*, 2000; Muir *et al.*, 2016; Veitch *et al.*, 2003), we first determined if α3β1 promotes mTLD/BMP-1 expression in keratinocytes. We compared mTLD/BMP-1 mRNA and protein levels between mouse keratinocyte (MK) cell lines that lack α3β1 (i.e., MKα3– cells, derived from an α3-null mouse), and the latter cells in which α3β1 expression was restored through stable transfection with human α3 (i.e., MKα3+ cells) (DiPersio *et al.*, 2000; Iyer *et al.*, 2005). Indeed, both mTLD/BMP-1 mRNA and protein were expressed at significantly greater levels in cells expressing α3β1, as determined by RT-PCR (Fig. 1a), qPCR (Fig. 1b), and western blot (Fig. 1c). Moreover, on a western blot the band with strongest reactivity to an antibody against mTLD/BMP-1 was consistent with the ~130 kD mTLD form of BMP-1 (Amano *et al.*, 2000) (Fig. 1c).

Next, we used RNA interference (RNAi) to determine whether mTLD/BMP-1 is required for LNγ2 processing in keratinocytes, which we previously showed was α3β1-dependent (Longmate *et al.*, 2014). MKα3+ cells (i.e., that express α3β1) were transfected with three distinct siRNAs that target transcripts from the *Bmp1* gene, or with a control siRNA that targets luciferase. Cells transfected with each mTLD/BMP-1-targeting siRNA displayed decreased mTLD/BMP-1 mRNA (Fig. 2a,b) and protein (Fig. 2c,d) compared to control transfected cells. Analysis of deposited matrix in each case revealed that suppression of mTLD/BMP-1 led to a reduction in the proportion of LNγ2 that was processed to the 105 kD form (Fig. 2e,f), demonstrating that this protease promotes efficient LNγ2 processing in MK cells. Together, these data demonstrate that integrin α3β1 promotes LNγ2 processing, at least in part, through regulation of *Bmp1* gene expression.

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Integrins α3β1 and α9β1 have opposing roles in regulating LNγ2 processing and mTLD/BMP-1 expression

We recently identified a role for the epidermal integrin α9β1 in suppression of α3β1 functions that promote paracrine stimulation of endothelial cells and wound angiogenesis (Longmate *et al.*, 2017). Although α9β1 is expressed in the epidermis of skin, it is lost upon culture of rodent and human keratinocytes (Choma *et al.*, 2004; DiPersio *et al.*, 2000; Singh *et al.*, 2009). Therefore, to investigate whether α9β1 has a similar cross-suppressive effect on α3β1-dependent LNγ2 processing, we stably re-expressed α9 in both MKα3– and MKα3+ cells to generate a panel of MK variants that express α9β1 and α3β1 in various combinations, as we described previously (Longmate *et al.*, 2017). Henceforth, these MK variants will be referred to as MKα3–/α9–, MKα3+/α9–, MKα3–/α9+, or MKα3+/α9+ to reflect their expression profiles of the α3 and α9 subunits (Longmate *et al.*, 2017). We previously showed that these MK variants display appropriate and similar cell surface expression of α9β1 and α3β1 (Longmate *et al.*, 2017). Although other groups have reported changes in other integrins in keratinocytes with natural or engineered deletion of the α3 subunit (Margadant *et al.*, 2009; Pazzagli *et al.*, 2017), we previously demonstrated that surface levels of other epidermal β1 integrins are not substantially altered in our MK model upon manipulated expression of α3, α9 or both together (Longmate *et al.*, 2017).

Consistent with our previous report that α3β1 promotes processing of the LNγ2 chain (Longmate *et al.*, 2014), expression of α3 alone (i.e., in the absence of α9) promoted both LNγ2 deposition and the proportion that was proteolyzed to the 105 kD processed form (Fig. 3a, b, compare α3–/α9– with α3+/α9–). In contrast, expression of α9 alone led to reduced LNγ2 processing, indicating that α9β1 has an independent suppressive effect on this proteolytic event (Fig. 3a,b; compare α3–/α9– with α3–/α9+). Interestingly, co-expression of α9β1 and α3β1 in MK cells suppressed the level of LNγ2 processing to baseline levels seen in MK cells that express neither integrin (Fig. 3a,b, compare α3+/α9+ with α3–/α9–). Furthermore, co-expression of α9β1 and α3β1 abolished α3β1-dependent upregulation of cellular mTLD/BMP-1 (Fig. 3c,d; compare α3+/α9– with α3+/α9+). Mass spectrometric analysis of secreted mTLD/BMP-1 revealed a pattern of relative expression among the MK cell variants that closely resembled that observed for LNγ2 processing, wherein α3β1 and α9β1 had counter-balancing effects (compare Fig. S1 to Fig. 3b).

Taken together, these observations are consistent with a cross-suppressive role for α9β1 over other α3β1 functions that we have reported (Longmate *et al.*, 2017), and they identify mTLD/BMP-1 as a mediator of α3β1-dependent LNγ2 processing. Of note, cellular levels of two BM/matricellular proteins, fibulin-2 and LNγ2 itself, were similarly counter-regulated by integrins α9β1 and α3β1 (Fig. S2), indicating that this suppressive role of α9β1 extends to α3β1-dependent expression of other matrix/matricellular proteins that modulate the tissue microenvironment.

Epidermal deletion of integrin α9β1 promotes LNγ2 processing *in vivo*

In order to determine whether α3β1 and α9β1 have similar counter-balancing effects on LNγ2 processing *in vivo*, we utilized mutant mice lacking α3β1 (α3eKO), α9β1 (α9eKO), or both integrins (α3/α9eKO) in epidermis (Longmate *et al.*, 2017). We previously reported

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that α3eKO mice display a persistent accumulation of unprocessed LNγ2 in 10-day wounds, compared to control mice, indicating that α3β1 promotes LNγ2 processing *in vivo* (Longmate *et al.*, 2014). As before, we utilized an antibody (anti-γ2L4m) directed against the globular L4 module of LNγ2 (depicted in Fig. 5c) to monitor its loss due to proteolysis during wound healing, as a readout for LNγ2 processing *in vivo* (i.e., positive staining indicates the presence of the unprocessed, precursor form of LN-332) (Longmate *et al.*, 2014; Sasaki *et al.*, 2001). As expected, wounds of α3eKO mice showed reduced LNγ2 processing, indicated by accumulation of unprocessed LNγ2. Levels of precursor LNγ2 in wounds from α3/α9eKO mice were comparable to that of wounds in control animals (Fig. 4a,b). In contrast, α9eKO mice showed reduced levels of unprocessed LNγ2 compared to controls (Fig. 4a,b), indicative of increased LNγ2 processing and consistent with the inhibitory role for α9β1 in LNγ2 processing that we observed *in vitro* (see Fig. 3).

mTLD/BMP-1 expression in post-wound epidermis is reduced in α3eKO mice but enhanced in α9eKO mice

Given that mTLD/BMP-1 mediated α3β1-dependent LNγ2 processing *in vitro* (Figs. 1 and 2), and wounds in mice that lack epidermal α3β1 displayed impaired LNγ2 processing (Fig. 4) (Longmate *et al.*, 2014), we next assessed mTLD/BMP-1 expression in wound epidermis of α3eKO mice. Reepithelialized wounds from α3eKO mice displayed reduced levels of mTLD/BMP-1, compared with reepithelialized wounds of control mice which showed mTLD/BMP-1 expressed in the basal keratinocyte layer (Fig. 5a,b). Conversely, wounds of α9eKO mice showed elevated mTLD/BMP-1 staining compared to control wounds (Fig. 5a,b), correlating with more complete LNγ2 processing that we observed in these wounds (see Fig. 4). Interestingly, mTLD/BMP-1 staining in α9eKO wounds extended into the suprabasal layers of the epidermis (Fig. 5a), possibly reflecting a delay in epidermal differentiation that was previously reported in these mice (Singh *et al.*, 2009). Of note, the BMP-1 antibody used in our immunohistochemistry studies is likely to have enhanced reactivity to the mTLD form of BMP-1 (see Materials and Methods). Overall, our data indicate opposing and balanced roles for integrins α3β1 and α9β1 in the regulation of BM maturation during wound healing, whereby α3β1 promotes mTLD/BMP-1 production and subsequent LNγ2 processing, while α9β1 coordinately tempers these processes (Fig. 5c).

DISCUSSION

Our previous findings have demonstrated that proteolytic processing of the LNγ2 chain, a hallmark of BM maturation, is impaired during adult wound healing in α3eKO mice, and in cultured α3-null keratinocytes (Longmate *et al.*, 2014). Until now, the mechanism through which α3β1 promotes LNγ2 processing has been unclear. Our current findings suggest that α3β1 promotes this processing event at least partly through induction of the *Bmp1* gene, which encodes mTLD/BMP-1, extracellular proteases that are known to mediate LNγ2 chain cleavage both *in vitro* and *in vivo* (Amano *et al.*, 2000; Muir *et al.*, 2016; Veitch *et al.*, 2003). Previous work from our group has demonstrated that integrin α3β1 can regulate mRNA expression/stability of Cox-2 in breast cancer cells and MMP-9 in keratinocytes through alternative splicing and alternative polyadenylation, respectively (Missan *et al.*, 2015; Subbaram *et al.*, 2014). While we have not yet determined the mechanism of α3β1-

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dependent *Bmp1* gene expression, we speculate that α3β1 may regulate mTLD/BMP-1 through similar posttranscriptional mechanisms. Indeed, several alternatively spliced transcripts of the *Bmp1* gene have been identified, including those encoding BMP-1 or mTLD (Takahara *et al.*, 1994), suggesting that the *Bmp1* gene may be prone to such regulation.

While our findings indicate an important role for mTLD/BMP-1 in α3β1-mediated LNγ2 chain cleavage, it is important to note that we have not ruled out potentially important roles for other proteases. For example, MT1-MMP and MMP-2 are two other proteases known to mediate LNγ2 processing, as reviewed elsewhere (Tzu and Marinkovich, 2008). Moreover, differential processing of the LNα3 chain by plasmin has also been shown to influence keratinocyte behavior (Goldfinger *et al.*, 1999; Goldfinger *et al.*, 1998). The involvement of several distinct proteases, and the potential context- and species-specific differences in the relative importance of these proteases, indicate that LN-332 processing is nuanced and complex (Tzu and Marinkovich, 2008).

Studies in β1 integrin-deficient mice or embryoid bodies have revealed essential roles for β1 integrins, such as α3β1, in proper BM formation in embryonic or adult tissues (Aumailley *et al.*, 2000; DiPersio *et al.*, 1997; Henry and Campbell, 1998; Longmate *et al.*, 2014), and mutations in the ITGA3 gene that encodes the α3 integrin subunit lead to compromised BM integrity and epidermal blistering in human patients with Junctional Epidermolysis Bullosa (Has *et al.*, 2012; He *et al.*, 2016; Nicolaou *et al.*, 2012). Like α3β1, α9β1 is an epidermal integrin that is upregulated post-wounding (Singh *et al.*, 2004). However, whether distinct epidermal integrins cooperate to mediate BM organization has not been investigated previously. Here, we speculated that coordinated activities of different integrins may be important for proper BM assembly. Like α3β1, integrin α9β1 is upregulated in the epidermis of healing wounds (Singh *et al.*, 2004) and we recently reported that α9β1 promotes blood vessel regression and vascular normalization at later stages of wound healing through its ability to suppress pro-angiogenic functions of α3β1 (Longmate *et al.*, 2017). Interestingly, our current findings indicate similarly opposing functions of α3β1 and α9β1 in BM maturation, where epidermal α3β1 promotes mTLD/BMP-1 expression and secretion and thus LNγ2 processing, while α9β1 inhibits these processes both *in vitro* and *in vivo*. We also found that addition of α9β1 to MK cells inhibited the ability of α3β1 to induce BM/matricellular proteins fibulin-2 and LNγ2, although in these cases we observed no statistically significant effects of α9β1 expression in the absence of α3β1. Together, these results indicate that in some cases α3β1 and α9β1 may also have independent and opposing effects on the same cellular function (e.g., LNγ2 processing), while in other cases α9β1 cross-suppresses α3β1-dependent function without having an obvious independent effect (e.g., expression of mTLD/BMP-1, fibulin-2 and LNγ2). In any case, counter-balancing roles for epidermal α3β1 and α9β1 appear critical for normal BM regeneration during wound healing, and for modification of the tissue microenvironment in general.

While integrin α3β1 has known roles in both stabilization and maturation of the cutaneous BM, these two processes appear separable. Indeed, previous findings from our lab indicate that α3β1 promotes BM stability in part through induction of fibulin-2, which was critical during adult wound-healing for epidermal adhesion since reepithelialized wounds of α3eKO

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mice blistered at the basement membrane zone (BMZ) (Longmate *et al.*, 2014). However, accumulation of unprocessed LNγ2 was not observed at sites of epidermal blistering in neonatal α3eKO mice, and unprocessed LNγ2 was detected even after blisters resolved in wounds of adult α3eKO mice (Longmate *et al.*, 2014), indicating that delayed LNγ2 processing was neither sufficient nor required to cause BM rupture that leads to blistering. Consistent with a clear separation between BM stability/epidermal adhesion and BM maturation, *Bmp1*-null mice die perinatally with defects in BM organization, but skin blistering was not reported (Burgeson and Christiano, 1997; Muir *et al.*, 2016; Suzuki *et al.*, 1996). In the current study, we observed that 10-day wounds of α3eKO mice have reduced mTLD/BMP-1 (Fig. 5) and display disorganized BM as evidenced by excess LN-332 staining beneath the BMZ (Fig. 4a, top panel), yet wound blistering resolves in these mice by 10 days (Longmate et al., 2014). We speculate that delayed LNγ2 processing might compensate for the blistering phenotype during wound healing in α3eKO mice, since the unprocessed LNγ2 short arm may allow for enhanced linkage to other ECM components that promote epidermal adhesion. Consistently, the unprocessed γ2 chain within LN-332 has been demonstrated to promote stronger cell adhesion rather than migration (Gagnoux-Palacios *et al.*, 2001).

Although it is not clear why the N-terminus of LNγ2 is proteolytically removed, retention of the full γ2 chain and the accompanying ECM interactions is not required to maintain a stable BM, since this region is absent from processed LN-332 in mature cutaneous BM (Aumailley *et al.*, 2003; Sasaki *et al.*, 2001). Given that unprocessed LN-332 supports adhesion (Gagnoux-Palacios *et al.*, 2001), while processed LN-332 may promote keratinocyte migration, we speculate that processed LNγ2 may poise the epidermis in a heightened “migration-ready” state to prepare skin for the next insult (Longmate *et al.*, 2014). A complex coordination of α3β1 and α9β1 may be required to properly maintain a balance between adhesion/stabilization and maturation of wound epidermis.

Thus far, failure to understand how different keratinocyte integrins function cooperatively to regulate wound healing has hindered the development effective integrin-targeting therapies. While many more studies are required to fully understand how different integrins cooperate during the process of wound healing, it is intriguing to conjecture that “cooperating” integrins not only collaborate towards a common goal of efficient wound healing, but that counter-regulatory roles for certain integrins may be critically important to regulate the extent and timing of distinct aspects of wound healing. Overall, our current findings indicate that the opposing functions of integrins α3β1 and α9β1 in healing wounds are important for proper LN-332 processing and BM maturation. Moreover, they further support that the coordinated functions of α3β1 and α9β1 control the keratinocyte secretome and, consequently, modification of the tissue microenvironment.

MATERIALS & METHODS

MK cells

The derivation of a panel of immortalized MK cells that express integrins α3β1 and α9β1, separately or together, was described in detail previously (Longmate *et al.*, 2017). MK cells were grown in keratinocyte growth medium consisting of Eagle's minimum essential

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medium (BioWhittaker, Walkersville, MD) supplemented with 4% fetal bovine serum (BioWhittaker) from which Ca^{2+} had been chelated, 0.05 mM $CaCl_2$, 0.04 μg/ml hydrocortisone, 5 μg/ml insulin, 2×10^{-9} M T3, 10 units/ml interferon-γ (Sigma, St Louis, MO), 10 ng/ml epidermal growth factor, 100 units/ml penicillin, 100 μg/ml streptomycin, and L-glutmamine (Invitrogen, Waltham, MA). MK cells were maintained at 33°C, 8% CO_2 on tissue-culture dishes coated with 30 μg/ml denatured rat tail collagen (BD Biosciences, Bedford, MA), as previously described (DiPersio *et al.*, 2000).

RT-PCR and qPCR

Complete RNA was isolated using the RNeasy plus isolation kit (Qiagen Valencia, CA), and quality and quantity was confirmed by NanoDrop. cDNA was generated using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). qPCR for *Bmp1* gene transcripts and β-actin were performed with iQ SYBR green Supermix (Bio-Rad) on a BioRad MyiQ PCR machine. The sequence and conditions for amplification of transcripts from the *Bmp1* gene were as follows: forward primer 5'-GACTCACGGCGGACTCTAAG-3'; reverse primer, 5'-CACTGGTGGATGTCACCTTG-3'; 95°C, 3 minutes, 1 cycle; followed by 95°C for 30 sec; 53°C for 30 sec; 72°C for 1 minute; for 22 cycles; 72°C for 2 minutes, 1 cycle. The sequence and conditions for β-actin were as follows: forward primer, 5'-GCCAGGTCATCACTATTGG-3'; reverse primer, 5'-AGTAACAGTCCGCCTAGAAGC-3'; 94°C, 3 minutes, 1 cycle; followed by 94°C for 1 minute; 58°C for 1 minute 30 sec; 72°C for 1 minute 30 sec; for 18 cycles; 72°C for 2 minutes, 1 cycle.

Immunoblot

Whole cell lysates were prepared in non-reducing cell lysis buffer (Cell Signaling Technology, Beverly MA) and protein concentrations determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Equal concentrations of protein were resolved by non-reducing 7% SDS/PAGE and assayed by immunoblot at the indicated dilutions of antibody: anti- mTLD/BMP-1 (1:200, ThermoFisher Scientific, Rockford, IL); anti-LNγ2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA); anti-fibulin-2 (1:2000) (Pan *et al.*, 1993); or anti-ERK (1:1000, Santa Cruz Biotechology, Santa Cruz, CA). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1400, Cell Signaling, Danvers, MA) or donkey anti-goat IgG (1:1000, Santa Cruz Biotechnology), as appropriate. Chemiluminescence was performed using SuperSignal Kit (Pierce), then visualized using Bio-Rad ChemiDoc MP imaging system with Image Lab software (Bio-Rad, Hercules, CA).

siRNA transfection

Cells were plated in full MK medium onto non-coated tissue culture dishes 24 hours prior to transfection. Lipofectamine 2000 (Invitrogen) reagent was used to transfect 88nM of siRNA into MK cells. Three different *Bmp1*-targeting siRNA's (Sigma-Aldrich, The Woodlands, TX) were as follows: siRNA #1- 5'-GCCAUAUCCAGUCCCAA-3', siRNA #2-5'-CUCAAUCACCCAGACUGGA-3', siRNA #3-5'-GCUAUAUUGUAUUCACCUA-3'. A luciferase-targeting siRNA was used as a control. Three days later, cells were either (1) isolated for immunoblot or PCR analyses (see above), or (2) lifted from the dish with 0.2% trypsin and re-plated onto non-coated tissue culture dishes for matrix preparation (see below).

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Matrix Preparation

MK cells were seeded onto non-coated tissue culture dishes in MK media supplemented with 4 mM $CaCl_2$ to promote matrix processing, as described (Amano *et al.*, 2000). After three days of culture, cellular fractions were removed from dishes with 1mM EDTA and lysed for immunoblotting (see above). Matrix fractions were prepared as previously described (Longmate *et al.*, 2014). Briefly, following cell removal, the deposited matrix was scraped into DOC buffer (2% sodium deoxycholate, 20 mM Tris-Cl pH 8.8, and 2 mM each of PMSF, EDTA, iodoacetic acid, N-ethylmaleimide), as described (Wierzbicka-Patynowski *et al.*, 2004). The DOC-insoluble matrix fraction was solubilized in 4% SDS/reducing sample buffer, and equal volumes were assayed by immunoblot with anti-LNγ2 (1:200, Santa Cruz Biotechnology), followed by HRP-conjugated donkey anti-goat IgG (1:1000, Santa Cruz Biotechnology). Detection was performed as above (immunoblot).

Mass Spectrometry (MS) analysis of secreted protein

Duplicate samples of serum-free medium were conditioned for 24 hrs by MK cell variants then equal protein was analyzed by MS (Thermo Fisher Center for Multiplexed Proteomics, Harvard Medical School). MS spectra were searched using the SEQUEST algorithm against a mouse Uniprot composite database derived from the mouse proteome, and peptide spectral matches were filtered to <1% false discovery rate using the target-decoy strategy combined with linear discriminant analysis. There were 29 spectral counts for mTLD/BMP-1.

Mice

Epidermis-specific α3 knockout (α3eKO) mice, epidermis-specific α9 knockout (α9eKO) mice, and epidermis-specific α3/α9 double-knockout (α3/α9eKO) mice are homozygous for a floxed α3 allele ($Itga3^{flx/flx}$) and/or α9 allele ($Itga9^{flx/flx}$), and express a Cre recombinase transgene under control of the epidermal-specific keratin 14 promoter (K14-Cre), as described (Mitchell *et al.*, 2009; Singh *et al.*, 2009). PCR genotyping and confirmatory immunostaining for appropriate loss of integrin expression has been described (Mitchell *et al.*, 2009; Singh *et al.*, 2009). All mouse studies were approved by the Institutional Animal Care and Use Committee at Albany Medical College.

In vivo wounding and immunohistochemistry

Adult mice (6–10 weeks of age) were anaesthetized and shaved, and four full-thickness wounds were made on the back of each mouse using a sterile 4-mm biopsy punch, as described (Mitchell *et al.*, 2009). After allowing wounds to heal for 10 days, mice were euthanized by CO_2 narcosis and wounds were surgically excised. Wounds were frozen in OCT compound, and 10 μm sections were prepared for immunohistology (Albany Medical College Histology Services). For immunostaining, frozen sections were rehydrated in PBS with 0.2% Tween-20 for 10 minutes, blocked in 10% heat-inactivated goat serum and 5% milk in PBS for 1 hour, then stained with the following rabbit polyclonal antisera: anti-LN-332 (1:200; Abcam, Cambridge, MA); anti-LNγ2 L4m (1:1000) (Sasaki *et al.*, 2001); or anti-BMP-1 (1:100; Abcam, Cambridge, MA). The anti-BMP-1 antibody used here was derived from a synthetic peptide based on the carboxyterminal end of the mTLD form of BMP-1, and is likely specific to mTLD. The secondary antibody used was Alexa Fluor 594

goat anti-rabbit IgG (1:250; Molecular Probes, Eugene, OR). Images were collected on a Nikon Eclipse 80i using a Spot camera (Diagnostic Instruments, Sterling Heights, MI).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

α3eKO	integrin α3 epidermal knockout
α9eKO	integrin α9 epidermal knockout
α3/α9eKO	integrin α3/α9 double epidermal knockout
MK cells	mouse keratinocytes
BM	basement membrane
BMZ	basement membrane zone
mTLD/BMP-1	mammalian tolloid/bone morphogenetic protein 1
LNγ2	laminin-γ2.

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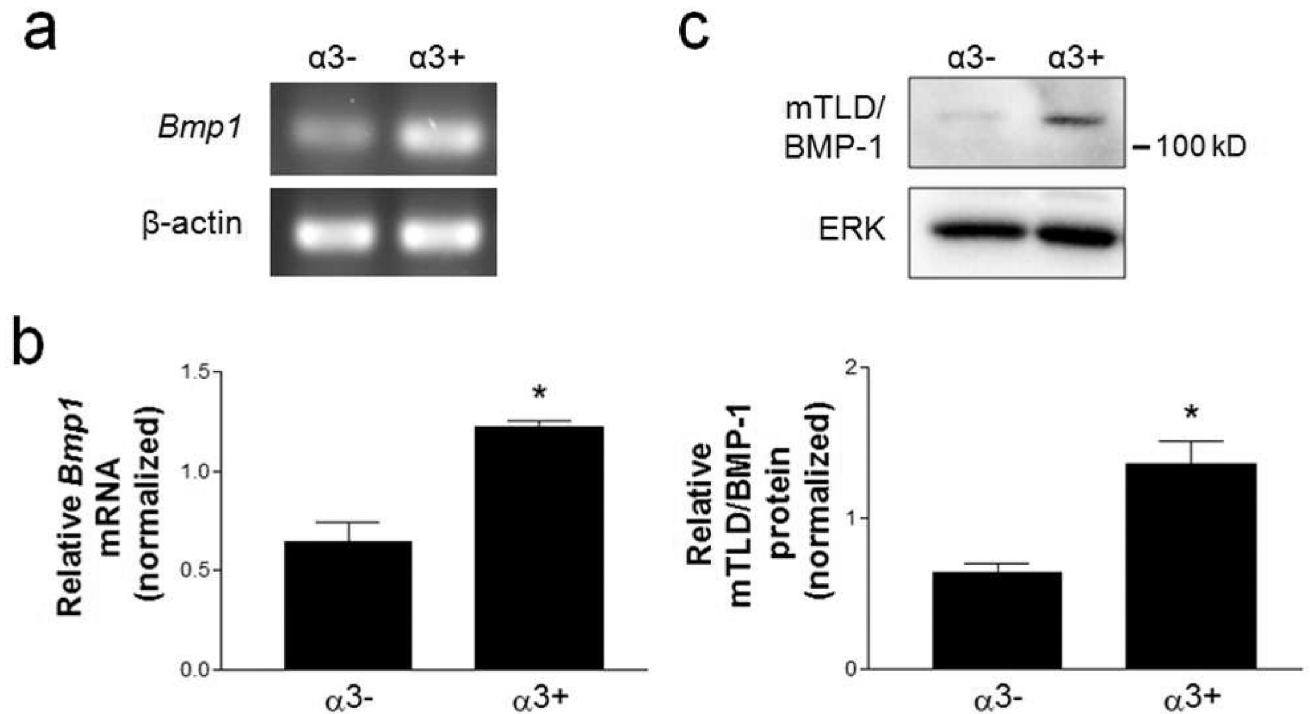

Figure 1.

Integrin α3β1 promotes mTLD/BMP-1 expression in mouse keratinocytes. (a,b) mRNA or (c) whole cell lystates were prepared from α3-null MK cells (α3−), or the latter cells with restored α3 subunit expression (α3+). (a) Representative images from RT-PCR for *Bmp1* gene products and β-actin. (b) qPCR analysis of relative *Bmp1* mRNA, normalized to β-actin mRNA. (c) Representative immunoblots of whole MK cell lysates are shown for mTLD/BMP-1 and ERK. Graph shows quantification of relative mTLD/BMP-1 protein, normalized to ERK, for 3 independent experiments. Data are mean ± SEM.; two-tailed t-test, *P<0.05.

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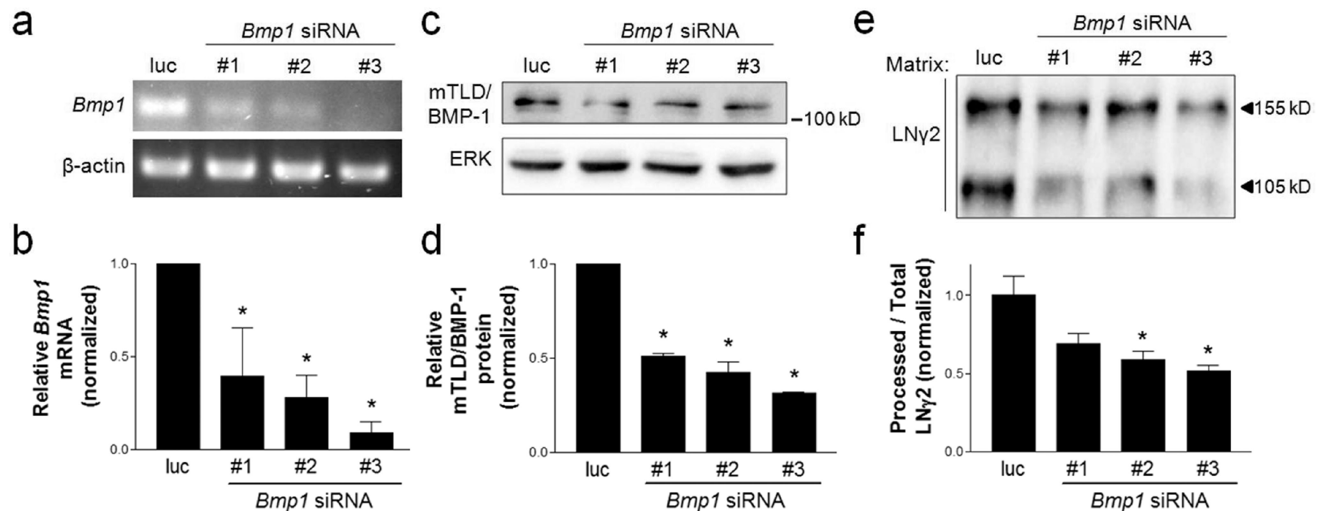

Figure 2.

siRNA-mediated suppression of the *Bmp1* gene reduces LNγ2 processing in α3β1-expressing keratinocytes. MKα3+ cells were treated with one of three distinct siRNAs that target *Bmp1* gene transcripts (#1–3) or a luciferase-targeting siRNA as a control (luc). (a) Representative RT-PCR and (b) quantification of relative *Bmp1* mRNA levels, normalized to β-actin mRNA. (c) Representative western blot of whole cell lysates and (d) quantification of relative mTLD/BMP-1 protein level, normalized to ERK protein. (e) Representative western blot of matrix preparations with anti-LNγ2. The unprocessed (155 kD) and processed (105 kD) forms of LNγ2 are indicated. (f) Quantification of processed LNγ2 as a proportion of total LNγ2, normalized to the daily mean to account for variability by day. All data are mean ± SEM; n=3; 1-way ANOVA; Dunnett's multiple comparison, *P<0.05.

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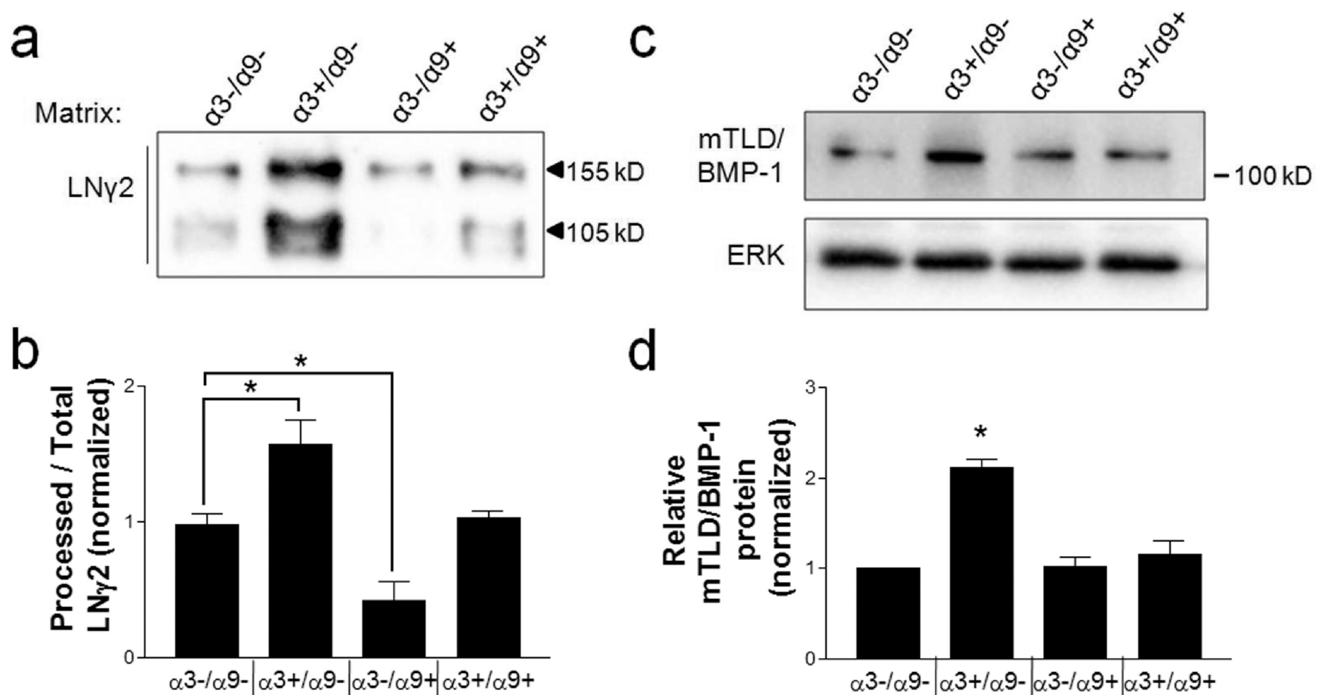

Figure 3.

α9β1 suppresses α3β1-dependent induction of LNγ2 processing and mTLD/BMP-1 expression. (a,b) ECM fractions or (c,d) whole cell lysates were collected from MK cell variants of the indicated integrin composition, which were cultured in high calcium to promote α3β1-dependent LNγ2 processing, as described (Longmate *et al.*, 2014). (a) Representative immunoblots of matrix preparations using anti-LNγ2. The unprocessed (155 kD) and processed (105 kD) forms of LNγ2 are indicated. (b) Quantification of processed LNγ2 as a proportion of total LNγ2, normalized to the daily mean to account for variability by day. (c) Representative immunoblots for mTLD/BMP-1 and ERK. (d) Quantification of relative mTLD/BMP-1 protein, normalized to ERK. Data are mean ± SEM; n 3; 1-way ANOVA; Newman-Keuls multiple comparison, *P<0.05.

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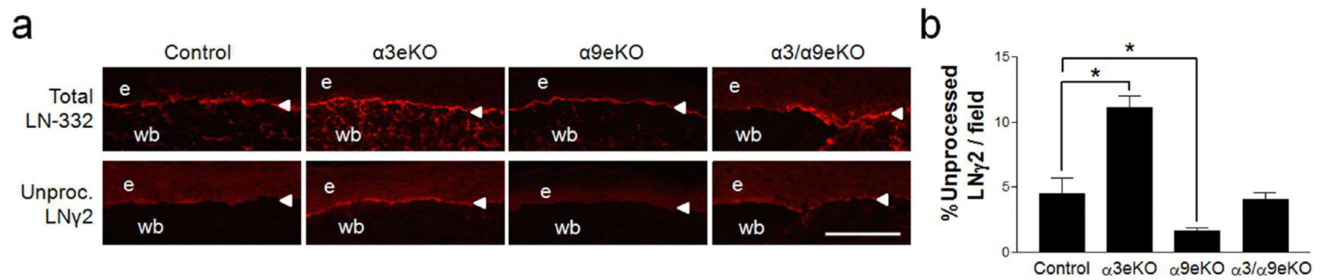

Figure 4.

Epidermal deletion of integrin α9β1 promotes LNγ2 processing *in vivo*. Cryosections of 10-day, fully reepithelialized excisional wounds were prepared from control, α3eKO, α9eKO, or α3/α9eKO mice and stained by immunofluorescence with anti-LN-332 (to detect total LN-332) or anti-γ2L4m (to detect the L4 module of unprocessed LNγ2 chain). e, epidermis; wb, wound bed; arrowhead, BMZ. (a) Representative images are shown. (b) Unprocessed LNγ2 was quantified for each genotype as a percent of positive staining per field of wounded skin; scale bar = 100 μm; mean ± SEM.; n 4 mice per genotype; 1-way ANOVA followed by Newman-Keuls multiple comparison, *P<0.05.

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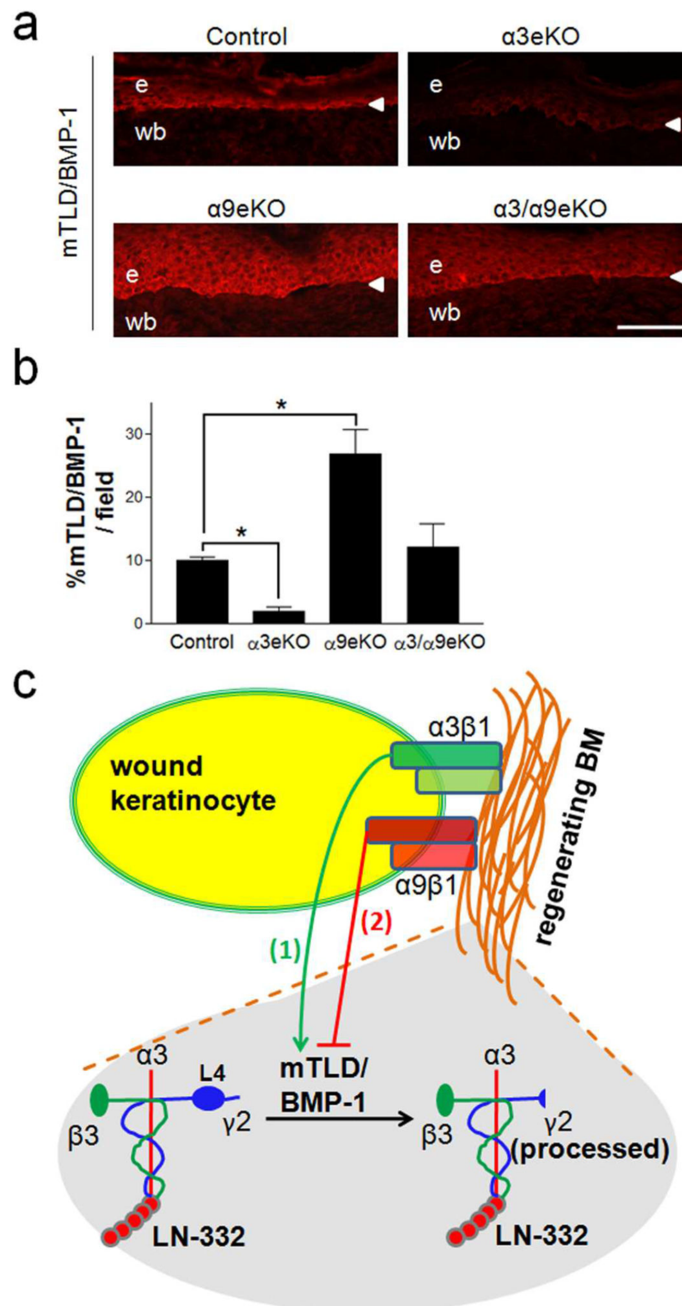

Figure 5.

α3eKO wound epidermis displays reduced mTLD/BMP-1 levels while α9eKO wound epidermis displays increased mTLD/BMP-1 levels. (a) Representative cryosections of 10-day reepithelialized excisional wounds were prepared from control, α3eKO, α9eKO, or α3/α9eKO mice and immunostained with anti-BMP-1. e, epidermis; wb, wound bed; arrowhead, BMZ. (b) mTLD/BMP-1 level was quantified for each genotype as percent positive staining per field of wounded skin; scale bar = 100 μm; mean ± SEM; n 5 mice per genotype; 1-way ANOVA followed by Newman-Keuls multiple comparison, *P<0.05. (c) Model illustrating our findings that (1) α3β1 promotes and (2) α9β1 inhibits expression

of mTLD/BMP-1, thereby regulating LNγ2 processing in regenerating BM. Note that the L4 module (detected by anti-γ2L4m) is present only in unprocessed LNγ2 (left).

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