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BMP1-like Proteinases Are Essential to the Structure and Wound Healing of Skin

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Abstract

Closely related extracellular metalloproteinases bone morphogenetic protein 1 (BMP1) and mammalian Tolloid-like 1 (mTLL1) are co-expressed in various tissues and have been suggested to have overlapping roles in the biosynthetic processing of extracellular matrix components. Early lethality of mice null for the BMP1 gene *Bmp1* or the mTLL1 gene *Tll1* has impaired *in vivo* studies of these proteinases. To overcome issues of early lethality and functional redundancy we developed the novel *BT^{KO}* mouse strain, with floxed *Bmp1* and *Tll1* alleles, for induction of postnatal, simultaneous ablation of the two genes. We previously showed these mice to have a skeletal phenotype that includes elements of osteogenesis imperfecta (OI), osteomalacia, and deficient osteocyte maturation, observations validated by the finding of BMP1 mutations in a subset of human patients with OI-like phenotypes. However, the roles of BMP1-like proteinase in non-skeletal tissues have yet to be explored, despite the supposed importance of putative substrates

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5. Ethical statement

The authors have nothing to declare.

of these proteinases in such tissues. Here, we employ *BT^{KO}* mice to investigate potential roles for these proteinases in skin. Loss of BMP1-like proteinase activity is shown to result in markedly thinned and fragile skin with unusually densely packed collagen fibrils and delayed wound healing. We demonstrate deficits in the processing of collagens I and III, decorin, biglycan, and laminin 332 in skin, which indicate mechanisms whereby BMP1-like proteinases affect the biology of this tissue. In contrast, lack of effects on collagen VII processing or deposition indicates this putative substrate to be biosynthetically processed by non-BMP1-like proteinases.

Keywords

bone morphogenetic protein 1 (BMP1) mammalian tolloid-like 1; skin; wound healing; collagen; biglycan; decorin

1. Introduction

Bone morphogenetic protein 1 (BMP1) is the prototype of a small family of structurally similar extracellular metalloproteinases, the BMP1/tolloid-like proteinases (BTPs), that perform morphogenetic roles, and are thought to activate growth factors and participate in forming the extracellular matrix (ECM) in a broad range of species [1]. The first demonstrated molecular role for a BTP was the biosynthetic processing of the major fibrillar collagens I-III by BMP1, via cleavage of C-propeptides from procollagen precursors [2]. Such processing is involved in producing mature collagen monomers capable of assembling into fibrils. Subsequently, studies have expanded the list of proposed BTP substrates to include additional ECM-related proteins biosynthetically processed to their mature/functional forms [1, 3, 4]. These include lysyl oxidase, an enzyme necessary for covalent crosslinking of elastin and collagen fibrils [5]; collagens V and XI, and the small leucine-rich proteoglycans (SLRPs) decorin and biglycan [6-9], all of which play roles in regulating collagen fibrillogenesis [10] [11-13]. Additional BTP ECM-related substrates have been reported to include collagen VII, involved in securing the epidermis to dermis [14], and laminin 332, a major component of epithelial basement membranes [15, 16]. In addition to roles in processing ECM components, BTPs have been implicated in the proteolytic activation of a small subset of transforming growth factor β (TGF β) superfamily members, including TGF β 1 [17] and ventralizing/osteogenic factors BMP 2/4 [18-21], via cleavage of extracellular antagonists. As TGF β signalling can induce the production of ECM components [22], TGF β activation is another potential mechanism by which BTPs may influence ECM formation.

There are four mammalian BTPs: BMP1 and mTLD (mammalian tolloid), which are encoded by alternatively spliced mRNAs from the same gene, and mTLL (mammalian tolloid-like) 1 and 2, which are encoded by distinct genes. Mice null for the *Bmp1* gene, which encodes BMP1 and mTLD, are perinatal lethal from defects that include failure to close the ventral body wall [23]. Mice null for the mTLL1 gene *Tll1* are embryonic lethal at E13.5, from cardiovascular defects [24]. *Bmp1*^{-/-} embryos have abnormal “barbed” collagen fibrils, consistent with possible diminished processing of putative BTP substrates [23]. In contrast, *Tll1*^{-/-} embryos have normal appearing collagen fibrils [24]. However,

although *Tll1*^{-/-} mouse embryo fibroblasts (MEFs) show no apparent deficits in processing of the putative BTP substrates tested, *Bmp1*^{-/-}; *Tll1*^{-/-} doubly null MEFs showed a reduction in proteolytic processing of probiglycan and procollagen I that was more pronounced than that found in MEF cultures null for *Bmp1* alone [6, 9]. These last results suggested some overlap/redundancy in function of products of the two genes. The possibility of functional overlap is reinforced by the findings that *Tll1* and *Bmp1* are co-expressed in a broad range of tissues [20, 25, 26] and share activity against a number of substrates in *in vitro* assays [3]. Unfortunately, the early lethality of *Bmp1*^{-/-} and *Tll1*^{-/-} mice has precluded study of *in vivo* roles of products of the two genes, either separately or in concert, in tissue homeostasis and disease.

In contrast to *Bmp1* and *Tll1*, the mTLL2 gene *Tll2* seems limited in its range of expression (developing muscle), and the phenotype of *Tll2*^{-/-} mice is limited to a mild muscle phenotype [27]. In addition, mTLL2 shows low or absent levels of activity against many ECM-related substrates *in vitro*. Thus *Tll2* is unlikely to be additive/redundant with *Bmp1* and *Tll1* in the cleavage of most tissue substrates.

To avoid the barriers of *Bmp1*^{-/-} and *Tll1*^{-/-} lethality and issues of functional redundancy, we recently developed a novel mouse strain with floxed *Bmp1* and *Tll1* alleles and a global, inducible Cre-ERT transgene (the *BT*^{KO} mouse), thus permitting simultaneous postnatal ablation of the two genes in all tissues [28]. These mice had a skeletal phenotype that included elements of osteogenesis imperfecta (OI) and osteomalacia, and deficits in osteocyte maturation that correlated with diminished proteolytic processing in bone of collagen I and dentin matrix protein 1 (DMP1)[28], the latter a protein involved in ECM mineralization and osteocyte maturation [29]. These observations demonstrated the importance of BTP activity, and the processing of these two substrates by BTPs, in bone. Moreover, the relevance of these findings to human health was validated by findings of *BMP1* mutations in a small subset of human patients with OI-like phenotypes [30-35]. However, possible BTP roles in non-skeletal tissues have yet to be explored, despite the supposed importance of putative BTP substrates in such tissues.

Here, we have employed *BT*^{KO} mice to investigate possible *in vivo* BTP roles in skin, a connective tissue rich in putative BTP substrates thought to be important to the biology of this tissue. *BT*^{KO} skin is shown to be markedly thinned and fragile, with abnormally dense packing of collagen fibrils, lower breaking energy (even when corrected for the thinness of the skin), and aberrant wound healing. Deficits are also shown for the processing of several putative BTP substrates in skin. However, the surprising finding of undiminished collagen VII processing in *BT*^{KO} skin suggests that the latter putative BTP substrate may instead be biosynthetically processed by non-BTP proteinases. Implications of the results and their relevance to human disease are discussed.

2. Results

2.1 Loss of BTP activity results in abnormal skin morphology

Gross examination revealed the dorsal skin of 20-week-old *BT*^{KO} mice to be noticeably thinner than that of controls (*Bmp1*^{flox/flox}; *Tll1*^{flox/flox} mice treated with tamoxifen, but

lacking a Cre transgene), a difference most apparent when pinching the scruff of the neck or other areas of dorsal skin. RT-PCR analysis of RNA extracted from dorsal skin of 20-week-old *BT^{KO}* mice showed this tissue to be doubly homozygous for excised *Bmp1* and *Tll1* alleles, with undetectable amounts of un-excised *Bmp1* and *Tll1* floxed sequences (Figs. 1A and B), consistent with loss of BTP activity in *BT^{KO}* skin subsequent to tamoxifen treatment.

Histological examination of dorsal skin from *BT^{KO}* and control mice revealed an 89% reduction in the average thickness of the hypodermal layer and a 39% reduction in the average thickness of the dermal layer of *BT^{KO}* skin, compared with controls (Figs. 1C, D and E). Masson's trichrome staining demonstrated *BT^{KO}* dermis to not only be thinner than control dermis, but to have a more compact appearance, with more densely packed collagen (Fig. 1D). Transmission electron microscopy (TEM) quantitation of collagen fibril diameters and numbers from mid dermis found *BT^{KO}* fibrils to have significantly decreased diameters (Figs. 2B and D, *BT^{KO}* 79±9.2 nm) compared to controls (Figs. 2A and C, 85±8.7 nm, mean ±standard deviation, *P*<0.01) and to be increased in numbers per unit area, compared to controls (*BT^{KO}* 4518 vs controls 3946 fibril counts, from 4 animals per genotype × 8 images per animal, as described in Experimental Procedures), consistent with increased density. TEM was also able to show a higher density of ECM in *BT^{KO}* dermis, with less free space between bundles of collagen fibers, than in control samples (Fig. 2E). Also noted was a dramatic reduction in the thickness of the more loosely organized papillary dermal layer in *BT^{KO}* mice, which would contribute to the overall increase in collagen density (Fig. 2E).

2.2 *BT^{KO}* skin-related molecular changes

A number of known and putative BTP substrates are key components of the dermal ECM. Collagen I, and to a lesser extent, collagen III, are present in abundance in the dermal layer. To begin examining the role of BTPs in the biosynthetic processing of these proteins in skin, dermal fibroblasts were isolated from *BT^{KO}* and control skin and conditioned media of these cells were examined by immunoblotting with an antibody (LF-67) against the C-telopeptide of the $\alpha 1(I)$ chain, which recognizes procollagen I, mature collagen I monomers, and all processing intermediates. Results revealed increased levels of pC $\alpha 1(I)$ (a processing intermediate in which the C-propeptide has been retained, but the N-propeptide has been cleaved) and an accompanying decrease in pN $\alpha 1(I)$ (a processing intermediate in which the N-propeptide has been retained, but the C-propeptide has been cleaved) in the conditioned media of *BT^{KO}* fibroblasts (Fig. 3A). In addition, immunoblotting with an antibody (LF-69) to the C-propeptide of procollagen III revealed increased levels of procollagen III and of pC $\alpha(III)$, and decreased levels of free (cleaved) type III collagen C-propeptides in the conditioned media of *BT^{KO}* dermal fibroblasts (Fig. 3B), indicating a similar delay in type III procollagen C-propeptide cleavage. Thus, results from immunoblotting of dermal fibroblast media were consistent with the conclusion that BTPs are important to the biosynthetic processing of the two major fibrillar collagens of skin.

Immunofluorescent staining for type I collagen in *BT^{KO}* and control skin samples, employing antibody LF67, revealed an increase in relative fluorescent intensity of collagen I in *BT^{KO}* skin (Fig. 3C) consistent with the more densely packed collagen fibers in *BT^{KO}*

skin observed by trichrome staining (Fig. 1D) and TEM (Fig. 2). In addition, and in accordance with the reduction of type I collagen C-proteinase activity seen in conditioned media (Fig. 3A), immunofluorescent staining for the procollagen I C-propeptide, employing antibody LF-41, was intense in *BT^{KO}* skin, but was essentially undetectable in control skin (Fig. 3D). Consistent with the immunofluorescent data, immunoblot analysis of control and *BT^{KO}* skin extracts with anti-C-propeptide antibody LF-41 readily detected bands only in the *BT^{KO}* sample (Fig. 3E). Interestingly, these bands corresponded to both procollagen I and pCa1(I) forms, and to free C-propeptide.

Decorin, a small leucine-rich proteoglycan (SLRP) thought to be involved in the regulation of collagen fibrillogenesis and collagen fibril diameters, has been shown to be processed by BTPs in biochemical assays [8], and deficits in the processing of its N-propeptide have been reported in cultures of dermal fibroblasts from three patients with OI resulting from mutations in the *BMP1* gene [30]. Consistent with these previous results, immunoblots of the extracts of *BT^{KO}* and control skin biopsies, employing anti-decorin antibody (LF-113), which recognizes both pro- and mature forms of decorin, showed a slight decrease in the electrophoretic mobility of the decorin band in *BT^{KO}* samples (Fig. 4A), consistent with a deficit in trimming of the prodecorin N-propeptide. Interestingly, immunofluorescent staining for decorin with the same antibody revealed markedly less decorin present in *BT^{KO}* dermis relative to controls (Fig. 4C).

Biglycan, another SLRP, with functions thought to overlap those of decorin, has also been proposed as a substrate for BTPs [6]. Immunoblotting with anti-biglycan antibody (LF-159, which recognizes both pro- and mature forms of biglycan) of conditioned media from dermal fibroblasts isolated from *BT^{KO}* and control skin, showed decreased electrophoretic mobility of the biglycan band in *BT^{KO}* samples, consistent with a deficit in proteolytic trimming of the probiglycan N-propeptide. Moreover, immunoblotting with an antibody specific for the prodomain of biglycan (LF-104) verified this larger band in the *BT^{KO}* samples to be probiglycan (Fig. 4B). Thus, BTPs participate in the processing of probiglycan in skin and such processing is greatly reduced in *BT^{KO}* skin. While decorin staining had lower intensity in *BT^{KO}* than in control skin (Fig. 4C), staining intensity for biglycan was at similar levels in *BT^{KO}* and control skin (Fig. 4D). Although immunostaining of skin detected decorin only in dermis (Fig. 4C), immunostaining detected biglycan in both the dermis and epidermis (Fig. 4D). In addition, although immunostaining with antibody to the probiglycan N-propeptide (LF-104) provided no detectable signal in the dermis of either *BT^{KO}* or control mice, it provided signal in the epidermis of both *BT^{KO}* and control mice (Fig. 4E).

In addition to its ability to regulate collagen fibrillogenesis, decorin is capable of inhibiting TGF β activity [36]. As immunofluorescent staining suggested a dramatic decrease in decorin amounts in *BT^{KO}* skin (Fig. 4C), we examined the degree to which TGF β activity might be affected. However, immunohistochemical staining of the dermis for pSmad2/3, a marker of TGF β activity, was comparable between *BT^{KO}* and control mice (data not shown), suggesting that reduced BTP cleavage of decorin did not affect TGF β signalling in the dermis.

2.3 Delayed wound healing in *BT^{KO}* mice

To further investigate how loss of BTP activity might affect skin biology, full thickness wounds were excised in the subscapular region of *BT^{KO}* and control skin. Wounds were then splinted open to decrease wound contraction and increase healing via re-epithelialization, ECM deposition, and granulation tissue formation. Gross examination of wounds over the course of the experiment revealed that wound closure occurred significantly more slowly in *BT^{KO}* mice compared to controls (Figs. 5A and B). Histological examination 6 days after wounding revealed a delay in re-epithelialization in *BT^{KO}* wounds (Figs. 5C and D), and by 15 days post-wounding there was a dramatic reduction in the thickness of newly formed dermal tissue in *BT^{KO}* wounds (Figs. 5E and F).

While there was no difference in immunohistochemical staining for collagen I between the newly formed ECM of *BT^{KO}* and control wounds (Fig. 6A), staining for the collagen I C-propeptide was only apparent in *BT^{KO}* wounds (Fig. 6B), the latter being consistent with the results in unwounded skin (Fig. 3D). As with type I collagen, loss of BTP activity did not appear to affect the amount of total decorin (Fig. 6C) or biglycan (Fig. 6D) in newly healed wounded tissue. While immunohistochemical staining for the prodomain of biglycan was present in the epidermis of both control and *BT^{KO}* wounds, probiglycan was also present in the stroma of *BT^{KO}* wounds, but absent from the stroma of controls (Fig. 6E), thus providing further evidence that probiglycan is a substrate of BTPs in skin.

At 15 days post wounding, *BT^{KO}* healing wounds were significantly thinner than controls, suggesting that less new ECM was being deposited into the wound beds of *BT^{KO}* mice. Myofibroblasts significantly contribute to the secretion of ECM components during wound healing and TGF β is thought to play important roles in the differentiation, recruitment and survival of myofibroblasts [37]. As BTPs are implicated in activating TGF β under certain circumstances [17], we were interested in whether delayed healing and reduction in ECM deposition in *BT^{KO}* wounds might be associated with decreased TGF β activity and/or a reduction in myofibroblast numbers. However, immunohistochemical staining for pSmad2/3 revealed no difference in TGF β activity between *BT^{KO}* and control wounded areas (Fig. 6F), while staining for α -smooth muscle actin, a myofibroblast marker [38], showed a modest increase in myofibroblast numbers in *BT^{KO}* wounds (Fig. 6G). Together these data indicate that any decreases in ECM deposition during wound healing in *BT^{KO}* mice are not due to decreases in TGF β activity or in ECM-producing cells.

2.4 Loss of BTP activity affects laminin 332, but not collagen VII processing/deposition at the dermal-epidermal basement membrane zone

Collagen VII is the major, if not sole protein component of anchoring fibrils, which mediate attachment of the epidermal basement membrane to the underlying dermis [39]. Collagen VII is synthesized as a precursor with a C-terminal NC2 prodomain that must be proteolytically removed before mature collagen VII monomers can self-associate to form mature, stable anchoring fibrils [40-42]. In previous studies, we found that although the NC2 domain can be cleaved by BTPs *in vitro*, procollagen VII processing appeared to be undiminished in *Bmp1^{-/-}* mouse embryos, suggesting that the *Tll1* product mTLL1 might be responsible for procollagen VII cleavage in skin, or at least capable of compensating for

loss of *Bmp1* in provision of this activity [14]. In the present study, immunoblotting with an antibody that recognizes both uncleaved procollagen VII and mature collagen VII detected similar levels of cleaved collagen VII, and similar small amounts of uncleaved procollagen VII, in *BT^{KO}* and control skin (Fig. 7A). In addition, immunofluorescent staining showed no apparent diminution or discontinuities in collagen VII deposition at the dermal-epidermal junction of uninjured *BT^{KO}* skin (Fig. 7B), or in healing *BT^{KO}* skin 6 days (Fig. 7C) or 15 days (Fig. 7D) post-wounding.

Laminin 332 is a major component of the anchoring filaments within the dermal-epidermal junctional basement membrane, and is important to stabilizing the attachment of epithelial hemidesmosomes to the epidermal basement membrane. Previous studies have provided evidence that the $\gamma 2$ and $\alpha 3$ chains of laminin 332 are biosynthetically processed by BTPs [15, 16]. In the present study, we found that although deposition of total laminin 332 is similar at the dermal-epidermal junctions of *BT^{KO}* and control skin 15 days post-wounding, use of a monoclonal antibody that recognizes only the normally cleaved portion of the $\gamma 2$ chain detected unprocessed laminin $\gamma 2$ chains deposited at the dermal-epidermal junctions of *BT^{KO}*, but not control, skin (Fig. 7E-G).

2.5 Effects of BTP activity loss on the biomechanical properties of skin

To ascertain possible biomechanical effects of BTP activity loss on the biomechanical properties of skin, skin samples from 17-20-week-old *BT^{KO}* and control mice were quantitatively compared for elastic modulus, tensile strength and toughness, via tensiometry. Mechanical testing revealed that dorsal skin breaking energy (toughness), with analysis that included correction for the thinner dorsal skin of *BT^{KO}* mice, was ~50% lower ($P < 0.0038$, Mann-Whitney U-test) in *BT^{KO}* mice than in controls (Fig. 8). Differences in tensile strength and Young's modulus did not reach significance. The lower breaking energy of *BT^{KO}* skin suggests reduced capacity of *BT^{KO}* dermis for plastic (post-yield) deformation prior to the point of definitive rupture.

3. Discussion

In this study, we employed the novel *BT^{KO}* mouse, with floxed *Bmp1* and *Tll1* genes and a ubiquitously expressed Cre transgene, to achieve conditional knockdown of BTP activity, thus overcoming barriers of early *Bmp1^{-/-}* and *Tll1^{-/-}* lethality, and functional redundancy. This enabled us to explore the *in vivo* roles of BTPs in skin. Loss of BTP activity is shown to markedly affect skin morphology, with pronounced reduction in thickness of both hypodermal and dermal layers. Additionally, *BT^{KO}* dermal collagen fibrils were of thinner diameter and more densely-packed than those of controls. While changes to the dermis are likely the direct result of lost BTP activity, it should be noted that the reduced thickness of the ordinarily adipocyte-rich hypodermis is congruent with our previous observations of a generalized decrease in the white adipose tissue of *BT^{KO}* mice [28]. However, it should also be noted that mice doubly null for decorin and biglycan show similarly reduced and atrophied hypodermis [13]. Thus, as both decorin and biglycan biosynthetic processing are disrupted in *BT^{KO}* mice, properly processed versions of these small proteoglycans may be key to the proper formation and/or maintenance of the hypodermis.

In addition to effects on skin morphology, loss of BTP activity is also shown to have pronounced effects on skin function. Thus, *BT^{KO}* skin is markedly deficient in wound healing ability, and uninjured *BT^{KO}* skin is shown to have decreased toughness, likely due to plastic deformation caused by irreversible ECM rupture or sliding at the fibrillar/molecular levels. These findings suggest that the subset of human patients with OI-like phenotypes due to mutations in the *BMP1* gene [30-35] may well have issues of skin fragility and impaired wound healing that will have to be monitored and managed.

Reduced procollagen I and III C-propeptide processing was evident for skin fibroblasts isolated from *BT^{KO}* mice, compared with those from controls. Moreover, although immunofluorescent staining for the procollagen I C-propeptide was absent in control dermal ECM, it was readily detectable throughout *BT^{KO}* dermal ECM (Fig. 3D), while immunoblotting of skin extracts detected procollagen I and pCa1(I) forms, and free C-propeptides, in *BT^{KO}*, but not control skin (Fig. 3E). Results are thus consistent with a marked deficit in the biosynthetic processing of skin procollagens. The persistence of procollagen I and pCa1(I) forms in *BT^{KO}* skin is consistent with our previous findings of such forms in *BT^{KO}* bone [43]. *BT^{KO}* bone, however, did not contain detectable free C-propeptides. These previous findings in *BT^{KO}* bone, plus our ability to immunostain the collagen fibrils of the ECM of *Bmp1/Tll1* doubly null mouse embryo fibroblast cultures with anti-C-propeptide antibody [44] previously led us to conclude that collagen I monomers with retained C-propeptides are capable of *in vivo* incorporation into collagen fibrils. This is in contradiction of previous *in vitro* fibrillogenesis assays that C-propeptide cleavage is necessary for incorporation of collagen monomers into growing fibrils [45]. As the immunofluorescent staining of *BT^{KO}* bone was due to procollagen I and pCa1(I) forms incorporated into fibrils, it is probable that the majority of staining with of *BT^{KO}* skin with anti-C-propeptide antibodies is also due, in large part, to procollagen I and pCa1(I) forms incorporated into fibrils. *BT^{KO}* dermal collagen fibrils were grossly normal in appearance, albeit more densely packed and with somewhat decreased diameters, compared to controls. Although, via TEM, we previously detected barb-like protrusions associated with retained C-propeptides on the collagen fibrils of *Bmp1^{-/-}* embryos and in cultures of *Bmp1^{-/-};Tll1^{-/-}* doubly null MEFs [9, 23, 44], barb-like protrusions were not apparent upon ultrastructural analysis of the collagen fibrils of *BT^{KO}* adult skin. Thus, the factors at play in the developmental/tissue contexts that determine the presence/absence of such barbs remain to be determined.

The detection of free C-propeptides, already cleaved from procollagen and pCa1(I) forms, in the extracts of *BT^{KO}*, but not control skin (or in *BT^{KO}* bone [43]) was surprising, and it is possible that it is responsible for some of the anti-C-propeptide staining of skin. We speculate that the absence of free C-propeptides in normal skin and in *BT^{KO}* bone is due to rapid and effective clearance of cleaved C-propeptides from both types of tissue. As there is no reason to believe that clearance of free C-propeptides from *BT^{KO}* skin would be slower than in these other tissues, we speculate that persistence of C-propeptides in *BT^{KO}* skin is from a continued replenishment via low level cleavage of C-propeptides from the retained procollagen I and pCa1(I) forms incorporated into fibrils. This slow release may be due to low levels of residual BTP activity in the knockdown skin. Alternatively, the slow release of C-propeptides in *BT^{KO}* skin, but not bone, may be due to the meprin α and β

metalloproteinases, which have been shown capable of cleaving procollagens I and III *in vitro* and which are at relatively high levels in skin, but not bone [46, 47]. Similarly, it remains to be determined whether the relatively low residual levels of C-propeptide processing of procollagens I (Fig. 3A) and III (Fig. 3B), and of probiglycan processing (Fig. 4B) in *BT^{KO}* dermal fibroblast cultures are due to residual BTP activity or to other proteinases, such as the meprins.

The *BT^{KO}* skin collagen fibrils in which procollagen I and/or pCα1(I) forms are incorporated, albeit unmarked by barbs, nonetheless appear to have changed physical properties, as evidenced by their tighter packing and reduced diameters, compared with controls. These changed physical properties may, in turn, reflect changed surface properties resulting from retained C-propeptides, consequently resulting in deficits in fine control in collagen fibril lateral associations and growth. The *BT^{KO}* collagen fibrils also appear to have inferior mechanical properties, as reflected in the increased fragility/reduced toughness of *BT^{KO}* skin.

Decorin and, to a lesser extent, biglycan are the major SLRPs of skin and provide overlapping functions within dermis [13, 48]. As noted above, we show here that BTPs are normally responsible for processing these SLRPs in skin. Previously, human OI patients with *BMP1* mutations were shown to have delayed decorin processing in skin fibroblast cultures [30]. Present results suggest that such patients likely have deficits in dermal biglycan processing as well. Interestingly, in wounded skin probiglycan was detected in *BT^{KO}*, but not in control dermis, although it was present in both *BT^{KO}* and control epidermis. In contrast, in uninjured skin, although probiglycan was still detected in both *BT^{KO}* and control epidermis, it was not observed in either *BT^{KO}* or control dermis. Thus, although BTPs seem responsible for relatively rapid biosynthetic processing of probiglycan during wound healing, other proteinases may compensate over time in uninjured skin for loss of BTP probiglycan-processing activity. Decorin was observed in the dermis of both *BT^{KO}* and control wounded skin (Fig. 6C), and in the dermis of unwounded control skin, but not in the dermis of unwounded *BT^{KO}* skin (Fig. 4C). We speculate that prodomain retention may affect the long-term stability of decorin, perhaps by affecting its ability to be incorporated into the ECM, such that decorin with retained propeptide does not persist in unwounded *BT^{KO}* skin, but is degraded over time. Presumably, decorin with retained propeptide is detected in wounded *BT^{KO}* skin because of relatively recent synthesis. Decorin was not observed in the epidermis of either wounded or unwounded *BT^{KO}* or control skin.

Our observations of probiglycan, but not decorin, in epidermis are consistent with previous reports of the presence of biglycan but not decorin in epidermis [49-51]. However, our results also show at least some portion of this epidermal biglycan to be in the form of probiglycan. We previously showed the major BTP produced by keratinocytes, the major cellular component of epidermis, to be mTLD, which has reduced ability to cleave probiglycan compared to BMP1 [6, 16, 52]. Moreover, we have shown cultured keratinocytes to produce only an unprocessed, zymogen form of mTLD, and smaller amounts of unprocessed BMP1, which retain their prodomains and would thus be inactive. Lack of epidermal BTP activity would be consistent with the presence of probiglycan in the epidermis of control mice. The purpose of inactive forms of BTPs and of unprocessed

probiglycan in epidermis remains to be determined. However, as in the case of other proteins found in the ECM in an inactive form, such as TGF β [53], pro-forms of BTPs and probiglycan in epidermal ECM may be available for rapid induction of activity/function in response to injury.

We have shown in previous *in vitro* assays that BTP processing of the prodomain of the SLRP osteoglycin potentiates ability of the latter to regulate collagen fibrillogenesis [54]. This suggests that processing of the related SLRPs decorin and/or biglycan is another, indirect, way in which BTP activity may affect formation/maintenance of the collagenous ECM of skin. Further studies are needed to determine the extent to which the decorin and biglycan prodomains affect the stability and activities of these proteins.

Delays in *BT*^{KO} wound closure and the dramatic reduction in ECM deposition in healed *BT*^{KO} wounds suggests that loss of BTP activity inhibits the re-epithelialization and rapid ECM formation necessitated by wound closure/healing. Evidence has previously been presented that BTPs may be involved in modulating TGF β signalling via cleavage of the extracellular antagonist LTBP (Latent TGF β Binding Protein) [17], and by cleavage of TGF β co-receptors betaglycan and CD109 [55]. TGF β can enhance ECM formation via recruitment of ECM-producing myofibroblasts and by up-regulation of many ECM-related genes, such as those encoding collagens I and III [22, 56]. We found TGF β activity, however, to be unaltered in *BT*^{KO} wounded and unwounded skin, compared to controls. Thus, BTPs may have little effect on TGF β signalling within wounded postnatal skin. Decreased ECM deposition during *BT*^{KO} wound healing is therefore likely due in large part to delays caused by incomplete processing of the precursors of ECM related proteins. Such roles for BTPs, as key regulators of ECM deposition in wounded skin, suggest these proteinases as candidate targets for anti-fibrotic drugs in the treatment of excessive formation of scar tissue, which is characterized by overly abundant collagen deposition.

Laminin 332 is a key component of the anchoring filaments that help keratinocytes adhere to the epidermal basement membrane [57], while collagen VII is the major, if not sole component of anchoring fibrils that mediate attachment of the epidermal basement membrane to the underlying dermis [39]. Previous studies have shown that the procollagen VII NC2 domain, cleavage of which seems necessary to anchoring fibril formation [42], can be cleaved *in vitro* by BTPs [14]. However, the seeming lack of reduced levels or sizes of collagen VII molecules at the dermal-epidermal junction in *Bmp1*^{-/-} mouse embryo skin previously suggested that mTLL1 activity might compensate for the loss of BMP1/mTLD in these embryos [14]. Here we show that this does not appear to be the case, as processing and deposition of collagen VII at the dermal-epidermal junction appears unaffected by disruption of both *Bmp1* and *Tll1* in *BT*^{KO} mouse skin. In fact, the data suggest that procollagen VII may well be processed by other, non-BTP proteinase(s) *in vivo*. Meprins α and β are possible candidate proteinases for the provision of such activity. In fact, due to the apparently low levels of persistent procollagen I C-propeptide cleavage in *BT*^{KO} skin (above), but high levels of procollagen VII NC2 cleavage, the meprins may play a relatively minor role in *in vivo* processing of the former, but an important role in processing of the latter.

Previous studies have also shown the N-terminal and LG45 domains of the laminin $\gamma 2$ and $\alpha 3$ chains, respectively, to be cleaved by BTPs *in vitro*, and that $\gamma 2$ processing was incomplete in *Bmp1*^{-/-} embryo skin [14, 16]. We confirm here that BTPs are indeed responsible for laminin 332 $\gamma 2$ chain cleavage *in vivo*, and show this to be the case in postnatal skin. As cleavage or retention of the $\gamma 2$ chain can apparently affect cell migration [57], laminin 332 $\gamma 2$ processing deficits may contribute to the delayed re-epithelialization of healing wounds observed in *BT*^{KO} mice.

4. Experimental Procedures

4.1 Mouse husbandry

Bmp1^{flox/flox}; *Tll1*^{flox/flox}; *Cre-ERT2* mice (in which inducible Cre expression is driven by the ubiquitin C promoter for ubiquitous Cre activity in tissues) were generated as described [28]. All animals in the study were housed and treated in accordance with NIH guidelines, using protocols approved by the Research Animal Resources Center of the University of Wisconsin-Madison under pathogen-free conditions with a 12-hour light / 12-hour dark cycle. Animals had *ad libitum* access to food and water. All mice used in this study were male.

4.2 Induced excision of *Bmp1* and *Tll1* in juvenile mice

To excise floxed *Bmp1* and *Tll1* sequences, tamoxifen (Sigma-Aldrich) was administered to *Bmp1*^{flox/flox}; *Tll1*^{flox/flox}; *Cre-ERT2* mice (*BT*^{KO} mice) and to *Bmp1*^{flox/flox}; *Tll1*^{flox/flox} mouse controls (lacking the *Cre-ERT2* transgene) via IP injection at a concentration of 100 mg/kg body weight (solubilized in 98% corn oil, 2% ethanol). Mice were injected with tamoxifen each day for 5 days at 4 and 5 weeks of age. At ~ 8 weeks of age the efficiency of gene excision was measured by PCR using genomic DNA from ear punch samples, and only mice showing >75% excision in the *Bmp1*^{flox} allele (95% of all tamoxifen-treated mice screened) were subjected to further analysis. Mice were sacrificed at 18 to 20 weeks of age for analysis of unwounded skin and at 10-12 weeks of age for wound healing experiments, and un-excised *Bmp1*^{flox/flox} or *Tll1*^{flox/flox} sequences were not readily detectable via PCR in the RNA extracted from these skin samples.

4.3 Splinted wound healing

Splinted wound healing was performed on 8- to 10-week-old *BT*^{KO} and control mice as described [58]. Briefly, all wounding procedures were performed under isoflurane anesthesia. The backs of mice were shaved and sterilized. 2 full-thickness wounds were created on the mid-back using a 5-mm biopsy tool (Integra Miltex). Circular, silicon splints with a 6 mm inner diameter were adhered around wound edges with cyanoacrylate glue (Elmer's Products) and fastened in place using 4 interrupted sutures. Wounds were covered by protective dressings for the duration of the experiment. Wound closure was analyzed with calipers by measuring 2 diameters per wound and using the average to estimate wound area. After sacrifice, wounded tissue was dissected for further analysis.

4.4 Histology

Mice were shaved and subscapular skin samples were removed and fixed in 4% paraformaldehyde at 4°C overnight. Fixed samples were embedded in paraffin and sectioned perpendicular to the epithelial surface (7 µm-thick sections). Wound tissue was dissected, fixed, and bisected prior to embedding. 7 µm serial sections from the center of the wounded area were used for further analysis. Hematoxylin and eosin and Masson's trichrome stains were used to evaluate general histomorphometry. Bright field images were captured using a Zeiss Axiophot 2 microscope with attached CCD camera. Re-epithelialization, wound thickness, and dermis/hypodermis thicknesses were quantified using Image J.

4.5 Immunohistochemistry and immunofluorescence

Following Heat-Induced Epitope Retrieval (HIER) in citrate buffer, sections were blocked in 10% BSA in PBS for 1 hour at room temperature. For decorin and biglycan staining, sections were treated with 0.1 U/ml chondroitinase ABC (Sigma) at 37°C for 2 hours to remove GAG chains. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 hour at room temperature. Primary antibodies used were: anti-pSmad2/3 (diluted 1:250, sc-11769-R, Santa Cruz), anti- α -SMA (diluted 1:8000, S0010, Epitomics); and anti-laminin 332 antisera (pKal) [16, 59] anti-laminin γ 2 N-terminal domain 6G6-C9 (kindly provided by Sarah Pogue of Teva Pharmaceuticals), anti- α 1(I) (LF-67), anti-pro- α 1(I) C-propeptide (LF-41), anti-decorin (LF-113), anti-probiglycan (LF-104), anti-biglycan (LF-159), and anti-collagen VII NC1 domain, all diluted 1:1000. LF-67, -41, -113, -104, and -159 [60] were kind gifts from Larry Fisher (National Institutes of Health, Bethesda, MD). Anti-collagen VII NC1 domain antibodies were the kind gift of Alexander Nyström and Leena Bruckner-Tuderman [61]. All primary antibodies, except for 6G6-C9, were rabbit polyclonal and have been described previously.

6G6-C9 is a rat monoclonal antibody that has not been previously described. Briefly, rats were immunized intradermally with gold particles bound to a proprietary DNA vector (Aldevron) encoding human laminin γ 2 amino acids 22-434. The DNA is designed to be taken-up by neighboring cells for peptide antigen expression on cell surfaces, thereby eliciting an immune response. Serum was screened for reactivity by FACS on cells transfected with the laminin γ 2 peptide antigen vector vs empty vector. Once a strong signal was achieved, lymphocytes were fused for generating hybridomas. Supernatants were screened by cell ELISA using the laminin γ 2 peptide-expressing cells vs empty vector-transfected cells. Hybridomas were subcloned and then screened in an ELISA assay employing laminin deposited by A431 cells or by MDA-MB-231 cells. 6G6-C9 recognizes A431-deposited laminin, which contains the G45 domain, but not MDA-MB-231-deposited laminin, which lacks the G45 domain. 6G6-C9 recognizes the 22-434 amino acid peptide from human and mouse.

Secondary antibodies used: Alexa 488-conjugated goat anti-rabbit IgG (Thermo Scientific), Alexa 594-conjugated goat anti-rat IgG (Thermo Scientific) and HRP-conjugated anti-rabbit IgG (BioRad), all diluted 1:1000. Fluorescent and bright field images were captured using a Zeiss Axiophot 2 microscope with attached CCD camera. For quantification of relative immunofluorescence intensity, the histogram function in Photoshop (Adobe) was used to

measure mean fluorescent intensity in 5 high magnification (400X) images per sample. All immunostaining was performed on at least 3 each *BT^{KO}* and control samples. Representative images are shown.

4.6 Transmission electron microscopy

For the electron micrographs of Figure 2E, subscapular skin was fixed in 1.5% glutaraldehyde/1.5% paraformaldehyde (Tousimis Research Corporation) in serum-free DMEM containing 0.05% tannic acid. Transmission electron microscopy (TEM) was performed on the skin samples using a standardized method, as previously described [62]. To determine skin collagen fibril diameters (Figs. 2A-D), skin samples were prepared for, and subjected to, TEM as previously described for tendon [28]. Fibril diameter analysis was obtained from pooled data from one skin sample from each of four mice of each genotype. Eight digital images from each skin sample were taken from non-overlapping areas at x60,000. Images were randomized and masked before fibril diameters were measured using a RM Biometrics-Bioquant Image Analysis System. A region of interest (ROI) of appropriate size was determined within the image so that a minimum of 80 fibrils was measured from each image. All fibrils in the ROI were measured per image. Fibril diameters were measured along the minor axis of the fibril cross section. Mid dermis fibril diameter measurements were pooled into groups by genotype.

4.7 Semi-quantitative RT-PCR

RNA was isolated from ground skin samples using TRIzol (Life Technologies), DNase (Promega), and further purification via phenol chloroform extraction. 1 µg of RNA was reverse transcribed following the manufactures instructions using oligo(dT) nucleotides and SuperScript (Invitrogen). Semi-quantitative RT-PCR to analyze excision of *Bmp1* and *Tll1* floxed alleles was performed using primers for *Tll1* (5'-CAGAGAGCCAATGTTCAAGCA-3', forward; and 5'-CTTGCAGGGTTTCTCCACAT-3', reverse) and *Bmp1* (5'-TGGATGAGGAGGACTTGAGG-3', forward; and 5'-GTGCTGTCTTGGAGGGTCTC-3', reverse) and *β-actin* (5'-TGGGTATGGAATCCTGTGGC-3', forward; and 5'-CCAGACAGCACTGTGTTGGC-3', reverse).

4.8 Dermal fibroblast culturing

Full thickness skin was excised from the axillary region of 10-12-week-old adult mice. Tissue was then minced with a pair of scalpels and digested overnight in 0.5 mg/ml type II collagenase (Worthington) in DMEM supplemented with 20% FBS at 5% CO₂ and 37°C. The digest was pelleted 5 minutes at 1000 g, the supernatant was removed, and the pellet was resuspended and plated in DMEM with 20% FBS.

For immunoblotting, cells were grown to 80-90% confluence, followed by treatment with 10 ng/ml TGFβ (R&D) and 75 µg/ml L-ascorbic acid (Sigma) for 24 hours. Cells were then washed three times with PBS to remove residual FBS before being serum starved in DMEM supplemented with 10 ng/ml TGFβ, 75 µg/ml L-ascorbic acid, and 40 µg/ml soybean trypsin inhibitor (Sigma). 24 and 48 hours post starvation, conditioned media were collected, pooled, and concentrated by centrifugation filters (Millipore).

4.9 Preparation of skin extracts

For analysis of collagen VII, skin was flash frozen with liquid nitrogen and then ground with a mortar and pestle under liquid nitrogen. 30 mg of skin powder was then boiled in 4X SDS-PAGE loading buffer containing 4 to 8 M urea. After centrifugation, the sample was diluted 1:4 with water prior to SDS-PAGE. For decorin and collagen I, skin was flash frozen with liquid nitrogen and then ground with a mortar and pestle under liquid nitrogen. Samples were then resuspended in T-PER reagent (ThermoFisher) with 1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, 1 mM *p*-aminobenzoic acid, and 10 mM EDTA, and sonicated 3 × 30 seconds on ice. Decorin samples were then subjected to treatment with chondroitinase ABC (Sigma) as described below for immunoblotting of conditioned media samples.

4.10 Immunoblotting

Concentrated conditioned skin fibroblast media and protein samples isolated from homogenized skin biopsies were used for immunoblotting. Human procollagen VII for immunoblotting was prepared as previously described [63]. For SLRP immunoblotting, samples were treated with 2.5 mU chordinase ABC (Sigma) at 37°C for 4 hours prior to immunoblotting. All samples were subjected to SDS-PAGE, and then transferred to nitrocellulose membranes, which were then blocked in 5% nonfat dry milk. Blots were probed with anti- α 1(I) (LF-67), anti-pro- α 1(I) C-propeptide (LF-41), anti-pro- α 1(III) C-propeptide (LF-69), anti-decorin (LF-113), anti-probiglycan (LF-104), and anti-biglycan (LF-159) [60]; as well as with anti-collagen VII (NC2-10, which recognizes both procollagen and mature collagen VII, a kind gift from Alexander Nyström and Leena Bruckner-Tuderman) [42], anti-PCPE1 (clone 7A11/1, Sigma) and anti- α -tubulin (clone DM1A, 05-829, Millipore) antibodies, all diluted 1:1000. Secondary antibodies used were IR800 conjugated anti-rabbit IgG and IR700 conjugated anti-mouse IgG (both diluted 1:15000, Li-Cor). Blots were visualized using an Odyssey FC Imager (Li-Cor). All immunoblots were repeated using at least 3 different *BT^{KO}* and control samples. Representative images are shown.

4.11 Biomechanical analysis

Tensiometric properties of moist skin strips (20 × 30 mm, trimmed to create a 5mm “waist” that provided a uniform breaking pattern) from the dorsal midline were evaluated using an Instron Tensiometer (Model 5542; Canton, MA). Mechanical properties (tensile stress, Young's modulus, and breaking energy) were calculated with Bluehill (v 2.32) software.

4.12 Statistical analysis

Where appropriate, data were analyzed using Fischer's 2-tailed t-test and $P < 0.05$ was considered significant. Tensiometric data were analyzed by the Mann-Whitney U-test. All data are recorded as average $\pm$ SEM.

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Abbreviations used

BMP1	bone morphogenetic protein 1
mTLL1	mammalian tolloid-like 1
OI	osteogenesis imperfecta
BTP	bone morphogenetic protein 1/tolloid-like proteinase
ECM	extracellular matrix
SLRP	small leucine-rich proteoglycan
TGFβ	transforming growth factor β
TEM	transmission electron microscopy
N-propeptide	NH ₂ -terminal propeptide
C-propeptide	COOH-terminal propeptide
pCa1(I)	processing intermediate that retains the C-propeptide but from which the N-propeptide has been removed
pNa1(I)	processing intermediate that retains the N-propeptide but from which the C-propeptide has been removed

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Highlights

- Loss of BMP1-like proteinase activity results in thin skin with densely packed collagen fibrils
- BMP1-like proteinases process collagens I and III, decorin, biglycan in skin.
- Ablation of BMP1-like proteinase activity delays wound healing in skin.
- Laminin 332, but not collagen VII, is processed by BMP1-like proteases during wound healing

Fig. 1.

Conditional disruption of *Tll1* and *Bmp1* in *BT^{KO}* skin, and morphological effects. (A) RT-PCR of total RNA from skin shows tamoxifen-induced excision of floxed *Bmp1* sequences. Bands of 243 and 811 bp correspond to *Bmp1^{Δ3-6}* (excised) and *Bmp1^{fl^{ox}}</sup>* (unexcised control) alleles, respectively. (B) RT-PCR of total RNA from skin shows tamoxifen-induced excision of floxed *Tll1* sequences. Bands of 179 and 544 bp correspond to *Tll1^{Δ6}* (excised) and *Tll1^{fl^{ox}}</sup>* (unexcised control) alleles, respectively. (C) Hematoxylin and eosin, and (D) Masson's trichrome staining of 18-20 week old control (Ctrl) and *BT^{KO}* skin

showed decreased dermal and hypodermal thickness in *BT^{KO}* mice. *BT^{KO}* dermal collagens (blue) stained more intensely than controls with Masson's trichrome stain. Epidermis, E, dermis, D, and hypodermis, H; Scale bars, 100 μ m. (E) Quantification of thickness of dermis and hypodermis. Measurements were taken from images of the histological sections and then measured using image J. The average dermal and hypodermal thickness for each mouse was calculated from 5 randomly selected measurements on each of 5 (non serial) sections for each mouse (25 measurements per mouse). P values: **<0.01, ***<0.001.

Fig. 2.

Ultra-structural analysis of dermis. (A-D) TEM images are shown of (A) control and (B) *BT^{KO}* mid-dermal collagen fibrils in cross section. Also shown are graphical representations of the frequencies of collagen fibril diameters used to quantify the Mean collagen fibril diameter for (C) control and (D) *BT^{KO}* mid dermis. Std Dev, standard deviation. (E) Upper panels, representative low magnification TEM images show reduced thickness of the papillary dermis (brackets), and increased compactness in the collagen fibrils in the reticular dermis of *BT^{KO}* skin. Lower panels, 4-fold higher magnification. Views of reticular dermis reveal dramatically more space (*) between collagen bundles in control (Ctrl) than in *BT^{KO}* skin.

Fig. 3.

Processing and distribution of the major dermal fibrillar collagens is affected by loss of BTPs. (A and B) Shown are representative immunoblots of conditioned media from dermal fibroblasts isolated from *BT^{KO}* and control (Ctrl) skin. (A) Use of anti-collagen I antibody (LF-67) shows increased levels of pCα1(I), and accompanying decreased levels of pNa1(I), processing intermediates in *BT^{KO}* fibroblast media, compared with control fibroblast media. (B) Use of anti-procollagen III C-propeptide antibody (LF-69) shows decreased procollagen III C-propeptide processing in *BT^{KO}* fibroblast media, compared with that of control fibroblasts. Molecular masses (in kDa) are indicated for protein standards. (C and D) Representative immunofluorescent staining (green) of skin samples from 18-20-week-old *BT^{KO}* and control mice, counterstained with DAPI (blue). Quantification of signal intensities from multiple immunostains (n=5) is shown to the right of each representative immunostain. A significant increase in staining intensity was seen in the *BT^{KO}* dermis compared to that of controls, employing either (C) anti-collagen I antibody (LF-67) or (D) anti-procollagen I C-propeptide antibodies (LF-41). Scale bars, 100 μm; P values: **<0.01, ***<0.001. ns, not significant. (E) An immunoblot is shown of extracts of control and *BT^{KO}* skin, probed with anti-procollagen I C-propeptide (LF-41).

Fig. 4.

Prodecorin and probiglycan are processed by BTPs in skin. (A) Shown are representative immunoblots of extracts from 18-20-week-old *BT^{KO}* and control (Ctrl) skin biopsies. Anti-decorin (DCN) antibodies (LF-113) show a decreased electrophoretic mobility for decorin in *BT^{KO}* samples, consistent with retention of the N-propeptide. (B) Representative immunoblots are shown of conditioned media from dermal fibroblasts isolated from *BT^{KO}* and control mice. Anti-biglycan (BGN) (LF-159) and anti-probiglycan prodomain (proBGN) (LF-104) antibodies show probiglycan to be present in conditioned media from

BT^{KO}, but not from control, dermal fibroblasts. Molecular mass (50 kDa) is indicated for a protein standard. (C-E) Representative immunofluorescent staining (green) of skin samples from 18-20-week-old *BT^{KO}* and control mice. All images were counterstained with DAPI (blue). Quantification of signal intensities from multiple immunostains is shown to the right of each representative immunostain. (C) There is marked reduction in the intensity of staining with anti-decorin antibody (LF-113) in *BT^{KO}* dermis, compared to that of controls. (D) However, no difference was detected in biglycan staining, with antibody LF-159, between *BT^{KO}* and control skin, in which signal was detected in both dermis and epidermis. For quantifications in (C) and (D), in each case n=5 immunofluorescent samples were observed from different mice. (E) Anti-probiglycan prodomain antibodies (LF-104) revealed no detectable staining in the dermis of *BT^{KO}* or control mice. High power magnification of area in white boxes highlights the probiglycan staining present in control and *BT^{KO}* epidermis. White scale bars, 50 μ m; red scale bars, 30 μ m; P values: **<0.01; ns, not significant.

Fig. 5.

Delayed wound healing due to deficits in BTP activity. (A) Representative images of BT^{KO} and control (Ctrl) healing wounds. (B) Quantification of open wound area shows wound closure to occur significantly more slowly in BT^{KO} than in control wounds ($n=10$ each for BT^{KO} and control groups). (C and E) Representative histological sections are shown of control and BT^{KO} wounds at 6 days (C) and 15 days (E) post wounding. High magnification images of wounded areas denoted by a black box are shown to the right. Sections were stained with hematoxylin and eosin. (D) Quantification of the lengths of epithelial tongues 6 days post-wounding indicated that re-epithelialization occurred significantly more slowly in

BT^{KO} wounds, compared to controls. (F) Average thickness of wounds at 15 days post-wounding is significantly decreased in *BT^{KO}* skin, compared to controls. Red arrows denote wound margins, black arrows denote edges of migrating epithelial tongues. (D and F) n=5 each for *BT^{KO}* and control groups. Red scale bars, 500 μ m; black scale bars, 20 μ m; P values: **<0.01, ***<0.001.

Fig. 6. Examination of candidate BTP substrates and TGF β activity in the newly formed ECM of healed wounds. Representative immunohistochemical staining (brown) is shown of the wounded area of BT^{KO} and control skin, 15 days post-wounding (each stained section shown is representative of $n=5$ stained sections from different mice). Antibodies used detected (A) collagen I, (B) procollagen I C-propeptide, (C) decorin, (D) biglycan, and (E) the N-propeptide of biglycan. (F) no differences were observed between genotypes in the proportion of pSmad2/3 positive cells in the newly formed stroma. (G) a modest increase in

the amount of α -smooth muscle actin was detected, indicating an increased number of myofibroblasts, in BT^{KO} wounds. Quantification of signal intensities from multiple immunostains (n=5) is shown to the right of representative immunostains in (F and G). Scale bars, 100 μ m (A-E) and 20 μ m (F and G); P values: **<0.01, ns, not significant.

Fig. 7.

Laminin 332 γ 2 chain processing and deposition, but not that of Collagen VII, are affected by loss of BTP activity. (A) A representative immunoblot of extracts of skin biopsies from 18-20-week-old *BT^{KO}* and control (Ctrl) mice, using anti-collagen VII NC2-10 antibody (which recognizes both mature collagen VII and procollagen VII), shows no decrease in levels of collagen VII deposited in *BT^{KO}* skin, and no decrease in procollagen VII processing, compared with controls. Molecular mass (250 kDa) is indicated for a protein standard, and human procollagen VII (Pro) was run in lane 1 as a standard. (B-D) Immunofluorescent staining (green) with the anti-NC1 domain antibody in (B) 18-20-week-old unwounded skin, (C) wounded skin 6 days post-wounding, and (D) wounded skin 15 days post-wounding - shows no differences in the distribution or intensity of signal for collagen VII between *BT^{KO}* and control skin. Insets in panels in (B) are 3-fold magnifications of areas within white boxes, highlighting the localization of collagen VII to the dermal-epidermal junction in both *BT^{KO}* and control skin. Immunofluorescent staining with (E) pKal polyclonal antibodies to whole laminin 332 (red), or with (F) monoclonal antibody 6G6-C9 to the normally cleaved N-terminal domain of the γ -chain (green) of 15-days-post wounding skin shows readily detectable signal for uncleaved γ chain in *BT^{KO}*, but not in control wounds; but similar total laminin 332 levels in *BT^{KO}* and control skin. Sections were counter stained with DAPI (blue). D, dermis; E, epidermis. Where used, scale bars are 40 μ m. In E-G dashed lines denote the boundaries between wounded skin and unwounded margins. pKal and 6G6-C9 stain the dermal-dermal basement membrane in both

unwounded and wounded skin, whereas pKal staining within unwounded dermis is of hair follicle basement membrane, and within wounded dermis appears due a turning over of basement membrane in the wound healing process.

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Fig. 8. BT^{KO} skin has markedly reduced breaking energy (toughness) compared to control skin. A box and whisker plot shows BT^{KO} dorsal skin to have significantly ($P < 0.0038$, Mann-Whitney U-test) reduced energy at break (BT^{KO} 0.0167 ± 0.0014 J vs controls 0.0253 ± 0.0017 J, mean $\pm$ standard error). For BT^{KO} mice and control mice $n=10$ and $n=20$, respectively.