Fibronectin matrix as a scaffold for procollagen proteinase binding and collagen processing

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ABSTRACT **The extracellular matrix (ECM) proteins fibronectin (FN) and type I collagen (collagen I) are codistributed in many tissues, and collagens have been shown to depend on an FN matrix for fibrillogenesis. Microscopic analysis of a fibroblast ECM showed colocalization of procollagen I with FN fibrils, and proteolytic cleavage of procollagen to initiate fibril formation was significantly reduced with inhibition of FN matrix assembly. We examined the role of FN matrix in procollagen processing by the C-propeptide proteinase bone morphogenetic protein 1 (BMP-1). We found that BMP-1 binds to a cell-assembled ECM in a dose-dependent manner and that, like procollagen, BMP-1 colocalizes with FN fibrils in the matrix microenvironment. Binding studies with FN fragments identified a binding site in FN's primary heparin-binding domain. In solution, BMP-1–FN interactions and BMP-1 cleavage of procollagen I were both enhanced by the presence of heparin, suggesting a role for heparin in complex formation during proteolysis. Indeed, addition of heparin enhanced the rate of procollagen cleavage by matrix-bound BMP-1. Our results show that matrix localization of this proteinase facilitates the initiation of collagen assembly and suggest a model in which FN matrix and associated heparan sulfate act as a scaffold to organize enzyme and substrate for procollagen processing.**

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INTRODUCTION

Collagen is the major insoluble fibrous protein in the extracellular matrix (ECM), conferring mechanical stability, tensile strength, and resilience to a wide range of tissues, making the proper synthesis, processing, and assembly of collagen vital to human health (Czarny-Ratajczak and Latos-Bielenska, 2000; Myllyharju and Kivirikko, 2001; Bateman *et al.*, 2009; Ricard-Blum, 2011; Boudko *et al.*, 2012; Steplewski and Fertala, 2012). Dysregulation of type I collagen, as well as the enzymes involved in its processing, has significant effects on the structure and mechanical properties of tissues and is the cause of a number of developmental abnormalities. Improper and excessive deposition of collagens is the basis for scar formation after tissue trauma and for fibrosis.

In the interstitial ECM, collagen I and the ubiquitous ECM glycoprotein FN are frequently found together. In fact, ECM deposition of collagens I, III, and IV, as well as of a number of other glycoproteins, is dependent upon the presence of a previously established FN matrix (McDonald *et al.*, 1982; Velling *et al.*, 2002; Sottile *et al.*, 2007; Singh *et al.*, 2010; Miller *et al.*, 2014). Because many of these proteins have FN-binding sites, the role of FN matrix as a foundation for further assembly may depend on its ability to directly interact with other ECM proteins. In support of this idea, a matrix assembled with FN that lacks its collagen/gelatin-binding domain was deficient in supporting collagen fibril formation (Sottile *et al.*, 2007). These findings indicate that type I collagen binding to FN is an integral part of collagen fibrillogenesis. However, other steps in addition to tethering to the ECM are needed for the formation of collagen fibrils.

Assembly of collagen requires proteolytic processing of procollagen by distinct proteinases ADAMTS-2 and bone morphogenetic protein 1 (BMP-1) that cleave the N- and C-terminal propeptides,

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Abbreviations used: BMP-1, bone morphogenetic protein 1; BSA, bovine serum albumin; DOC, deoxycholate detergent; DTT, dithiothreitol; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin; GST, glutathione *S*-transferase; HS, heparan sulfate; IgG, immunoglobulin G; PBS, phosphate-buffered saline; pNcollagen, N-propeptide retaining collagen; rhBMP-1, recombinant human BMP-1; rhPro-COL1A1, mini recombinant human procollagen αI(I) homotrimer.

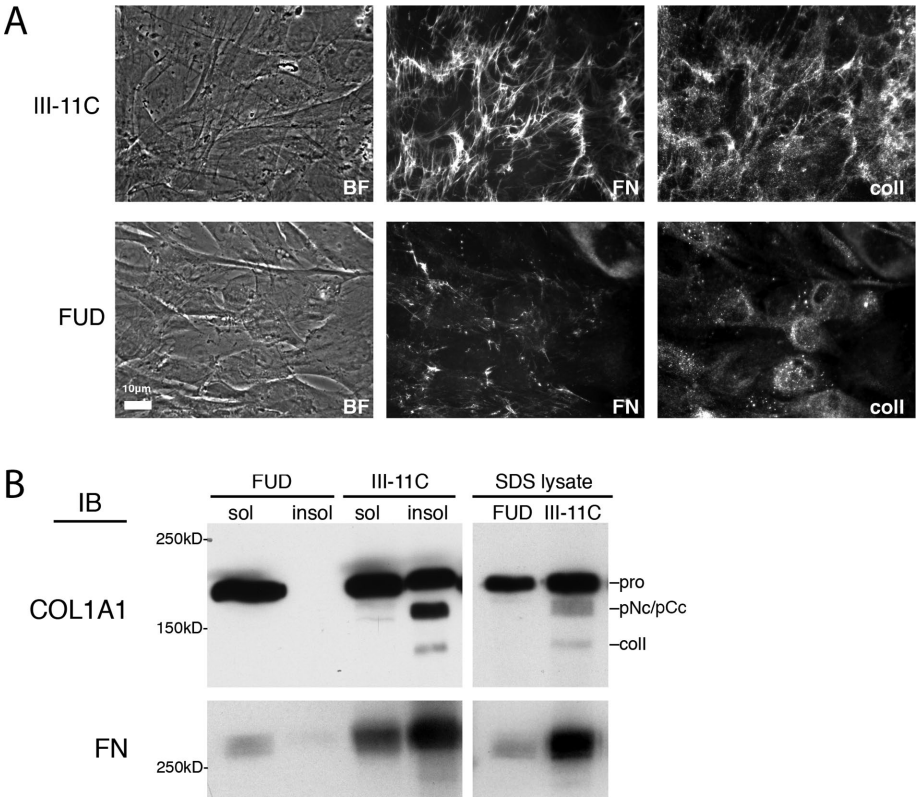

FIGURE 1: Fibronectin matrix assembly is necessary for proteolytic processing of procollagen I. (A) GM03349 human dermal fibroblasts were grown to confluence as shown in the phase images (BF) in the presence of control peptide (III-11C) or FUD peptide to inhibit FN matrix assembly. Cells were fixed and costained for FN and collagen I (coll) with antibodies hFN7.1 and PA2140-2, respectively, followed by the appropriate fluorescent secondary antibodies. Representative epifluorescence images of a single field are shown for each peptide treatment. Scale bar: 10 μm. (B) Cells grown and treated as in A were lysed in either DOC or SDS buffer. DOC-insoluble material was collected from DOC lysates by centrifugation and then solubilized by boiling in buffered SDS. Samples were separated by electrophoresis in a 5% polyacrylamide gel and immunoblotted with anti-COL1A1 (top) or anti-FN (R184) antibody. Locations of procollagen, pN- or pC-collagen, and cleaved collagen I are indicated (right); two bands can be detected in the pNc/pCc band in lysates from subconfluent cells. Molecular mass standards are indicated on the left.

respectively (Prockop and Hulmes, 1994; Canty and Kadler, 2005; Greenspan, 2005; Kadler *et al.*, 2008). BMP-1 and its enhancer PCPE-1 have been shown to bind to FN (Huang *et al.*, 2009; Weiss *et al.*, 2014), suggesting a mechanism in which FN matrix controls collagen assembly through interactions with processing enzymes. Proteolytic cleavage of the C-propeptide by BMP-1 is necessary for assembly of collagen I molecules and fibrillogenesis (Kivirikko and Myllyla, 1985; Mould *et al.*, 1990; Canty and Kadler, 2005). In contrast, collagen I fibrils that retain the N-propeptide (pNcollagen) have been detected in vivo, and fibrillar assemblies of pNcollagen I can be formed in vitro from purified protein (Fleischmajer *et al.*, 1981; Hulmes *et al.*, 1989). Therefore N-propeptide cleavage, and thus ADAMTS-2 activity, is not required to initiate fibrillogenesis.

The necessity for BMP-1 cleavage of procollagen I for fibrillogenesis and the ability of BMP-1 to bind to FN led us to question the role of the FN matrix in the molecular interactions that regulate BMP-1 activity and its localization within the ECM. BMP-1 is activated from its pro-form by furins in the secretory pathway (Leighton and Kadler, 2003). Cleavage of collagen primarily occurs in the extracellular space (Lee *et al.*, 1997; Gelse *et al.*, 2003; Greenspan, 2005), and analysis of developing tendon has shown BMP-1 localization within cell protrusions (Birk and Trelstad, 1986; Canty *et al.*, 2004; Canty-Laird *et al.*, 2012; Leslie, 2006). Other studies have observed BMP-1 near the cell surface, where FN is usually localized (Peters and Mosher, 1987; Christopher *et al.*, 1997; Norman *et al.*, 2003; Canty *et al.*, 2004; Singh *et al.*, 2010; Schwarzbauer and DeSimone, 2011). Therefore, this proteinase is positioned to participate in interactions with FN that may affect its ability to act on procollagen.

In this study, we examine the contributions of FN matrix to proteolytic processing of procollagen I. Biochemical and microscopic analyses of procollagen processing by BMP-1 are used to show a role for binding to FN matrix. Further, we establish a previously unidentified role for heparin/heparan sulfate (HS) in this process. Our data suggest a model for nascent collagen fibrillogenesis through procollagen processing on an FN matrix.

RESULTS

Dependence of procollagen I proteolytic processing on FN matrix

Primary human dermal fibroblasts (GM03349) assemble a matrix composed of fibrillar FN and type I collagen. Treatment of these cells with the bacterial peptide FUD, which binds to the assembly domain of FN (Maurer *et al.*, 2010), inhibits the formation of FN fibrils and eliminates collagen I assembly (Figure 1A). FUD treatment does not affect FN or type I collagen mRNA expression levels (Chiang *et al.*, 2009). These results confirm that, as with other cell types (Velling *et al.*, 2002; Sottile *et al.*, 2007), these fibroblasts require an FN matrix for collagen I deposition. During collagen fibril formation, N- and C-propeptides are cleaved by extracellular proteinases. To observe the effects of FN matrix on this proteolytic cleavage, we compared the collagen profiles from cells treated with FUD or a control peptide. Newly assembled FN fibrils are soluble in the detergent deoxycholate (DOC) but are converted to a DOC-insoluble form, allowing separation of these lysates into nascent and stable matrix fractions. Immunoblots of cell lysates treated with a control peptide (III-11C) show the presence of FN and of procollagen I in DOC-soluble and DOC-insoluble fractions (Figure 1B). Collagen cleavage products lacking one or both propeptides are evident in the DOC-insoluble matrix. In contrast, inhibition of FN assembly in FUD-treated cells results in a significant reduction of collagen in DOC-insoluble samples. Whole-cell lysates prepared with an SDS lysis buffer demonstrate the lack of procollagen proteolytic processing when FN assembly is inhibited (Figure 1B). Three distinct bands in the control sample represent procollagen I, pNcollagen or pCcollagen lacking either the C-propeptide or N-propeptide, respectively, and mature collagen I lacking both propeptides. Procollagen processing only in the presence of an FN matrix suggests that FN matrix may have a role in localizing procollagen and/or procollagen proteases to promote proteolytic cleavage and collagen fibrillogenesis.

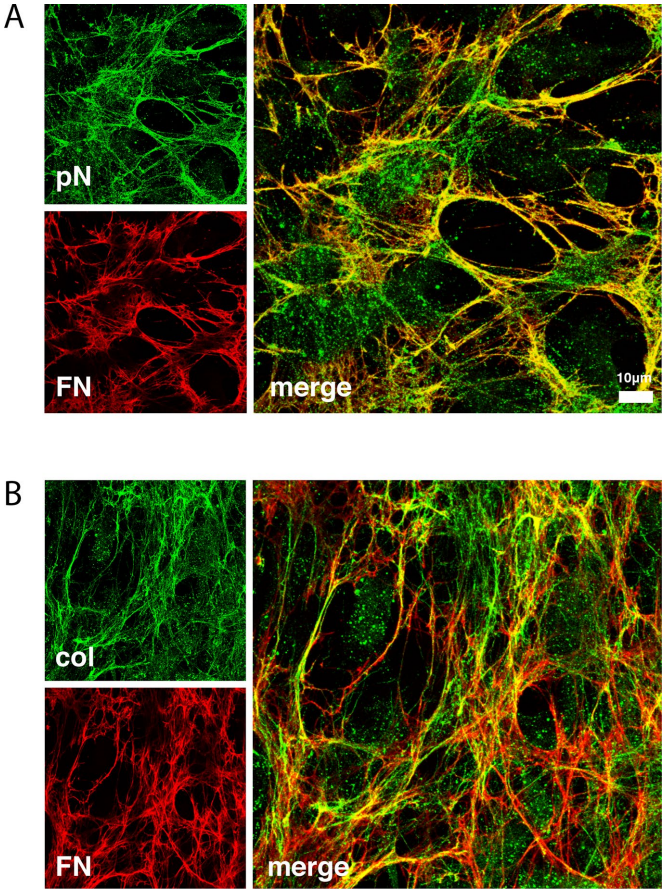

FIGURE 2: Procollagen colocalizes with FN fibrils. GM03349 cell cultures at confluence were coimmunostained for FN and either (A) pN collagen (pN) with anti–N-propeptide antibody or (B) collagen (col) with a polyclonal anti-COL1A1 antibody. Primary antibodies were detected with appropriate fluorescent secondary antibodies (FN, red; collagens, green) and confocal microscopy. Scale bar: 10 μm.

To investigate the localization of procollagen, we took advantage of the fact that cleavage of the C-propeptide but not the N-propeptide is needed for collagen to form fibrils (Fleischmajer *et al.*, 1981; Kadler *et al.*, 1987). Fibroblasts costained with antibodies against the N-propeptide of procollagen I and FN show a high degree of colocalization between fibrils of pNcollagen and FN (Figure 2A). This colocalization suggests that FN can participate in procollagen processing into collagen, whether through direct or indirect interactions. Immunostaining with an antibody that detects total collagen I shows a lower degree of colocalization with FN fibrils compared with procollagen staining (Figure 2B). The disparity in colocalization with FN fibrils between procollagen and collagen insinuates that, once cleaved, fibrillar collagen no longer needs to remain associated with FN. Coimmunostaining for total collagen and the procollagen N-propeptide support this claim, with a greater number of fibrils stained for total collagen than for pNcollagen (Supplemental Figure S1).

BMP-1 binding to the FN matrix

Localization of procollagen to FN fibrils implies that procollagen proteinases are associated with the ECM. The C-propeptide protease BMP-1 is known to have FN-binding activity (Huang *et al.*, 2009). For measurement of its interaction with an FN-rich ECM, a modified enzyme-linked immunosorbent assay (ELISA) was performed after incubation of recombinant human BMP-1 (rhBMP-1) at increasing concentrations with GM03349 cell cultures. Significant, dose-dependent binding of rhBMP-1 to the FN matrix was observed with maximal binding at 5 μg/ml (Figure 3A). For examination of the distribution of the proteinase in the matrix, fibroblasts were supplemented with rhBMP-1 and immunostained for BMP-1 and FN. rhBMP-1 addition was necessary, as we have not been able to detect endogenous BMP-1 in GM03349 cell cultures by immunostaining or immunoblotting. Immunofluorescence images of cultures stained with either an anti–BMP-1 antibody or an antibody against the His tag on the rhBMP-1 reveal that, like procollagen, BMP-1 also colocalizes with FN fibrils (Figure 3B). Both antibodies gave diffuse, speckled background staining on control cell cultures with no discernible localization along FN fibrils. For confirmation that rhBMP-1 binds to the ECM and not to cell surfaces, the rhBMP-1–binding experiment was performed with decellularized matrix prepared from GM03349 cells. The removal of cells was confirmed by phase microscopy (Supplemental Figure S2). Significant, dose-dependent binding of rhBMP-1 to the cell-free matrix was observed (Figure 3C). We have previously shown that FN is the predominant protein in cultured fibroblast matrix (Mao and Schwarzbauer, 2005). These results support the conclusion that BMP-1 associations with the ECM are through its binding to FN.

To probe direct interactions between BMP-1 and FN, we first confirmed that rhBMP-1 binds to FN. Using gelatin-Sepharose to capture FN, we found that the presence of BMP-1 in the pull-down was dependent on FN (Figure 4A, lanes 2 and 3). BMP-1 reportedly has multiple binding sites on FN (Huang *et al.*, 2009), so glutathione *S*-transferase (GST)-tagged recombinant FN fragments were used to identify sites of interaction. We found that BMP-1 binds to the HepII domain of FN (repeats III12-15) but not to the cell-binding domain (repeats III9-10) (Figure 4B). The efficiency of the pull-downs was confirmed by immunoblotting for the recombinant fragments using anti-GST antibodies (Supplemental Figure S3). HepII is the major heparin/HS-binding site on FN (Hynes, 1990; Barkalow and Schwarzbauer, 1991), and heparin is also known to bind to BMP-1 (Bekhouche *et al.*, 2010). Addition of heparin to FN and to fragment-binding assays shows that heparin promotes rhBMP-1 binding to the HepII domain of FN (Figure 4, A and B).

Proteolytic cleavage of procollagen I by BMP-1 in solution

To test whether heparin binding to BMP-1 affects processing of native procollagen, we treated GM03349 cell–conditioned medium containing cell-produced procollagen I with rhBMP-1. The concentration of rhBMP-1 was determined by titration, and in subsequent experiments, we used 0.5 μg/ml, which gave partial cleavage of procollagen I at 15 min (Supplemental Figure S4A). Conditioned medium was supplemented with rhBMP-1 alone or with rhBMP-1 plus heparin, and proteolytic processing of endogenous procollagen was monitored over time by immunoblotting with an anti–procollagen I N-propeptide antibody. Treatment with rhBMP-1 alone shows a time-dependent generation of the pNcollagen band and reduction in the procollagen I band (Figure 5A). After 15 min, about half of the procollagen was intact (Supplemental Figure S5A), and at the conclusion of the time course, detectable unprocessed procollagen remained in the absence of heparin. Supplementation with heparin significantly enhanced cleavage with no detectable procollagen after only 15 min (Figure 5B and Supplemental Figure S5A). Compared with no heparin at 15 min, this shows that heparin facilitates procollagen cleavage by BMP-1.

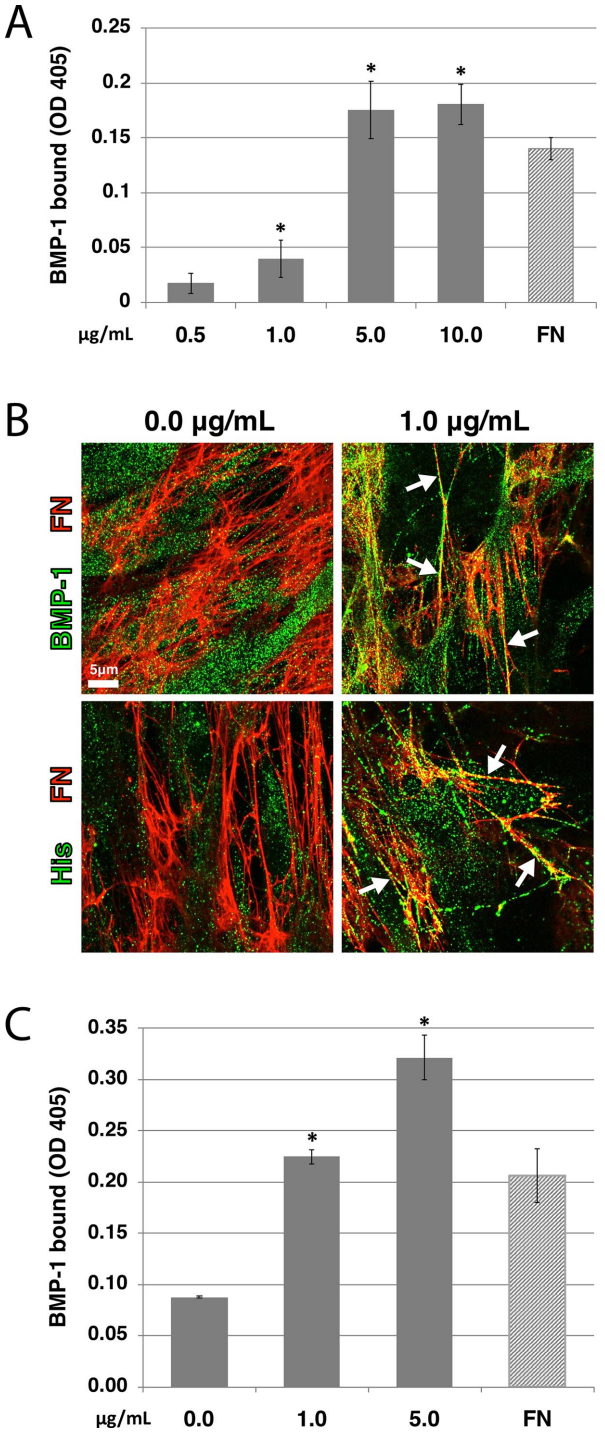

FIGURE 3: BMP-1 binds to the FN matrix and colocalizes with FN fibrils. (A) rhBMP-1 was incubated at increasing concentrations with GM03349 cell–assembled matrices. Binding was detected by ELISA with anti–BMP-1 antibody and measured through a colorimetric readout. The presence of FN was confirmed by ELISA with anti-FN antibody. Bars show the average of three experiments ± SEM, *, $p < 0.05$ compared with lowest BMP-1 concentration. (B) GM03349 cells at confluence were incubated with medium alone (left) or medium with 1.0 µg/ml rhBMP-1 (right) and then fixed and costained for FN with R184 (red) and BMP-1 (green). Antibodies against BMP-1 (top) or against the His tag on rhBMP-1 (bottom) were used. Nonspecific background signal was observed with anti–BMP-1 and anti-His antibodies in the absence of added rhBMP-1. White arrows indicate some regions of colocalization. (C) GM03349 cells grown past confluence in a 96-well plate were decellularized and cell-free matrices were incubated with rhBMP-1 at the indicated concentrations. Binding was detected by ELISA as described in A. Bars show the average of three experiments ± SEM *, $p < 0.05$ compared with no rhBMP-1 added. The cross-hatched bar confirms the presence of FN by ELISA with anti-FN antibody.

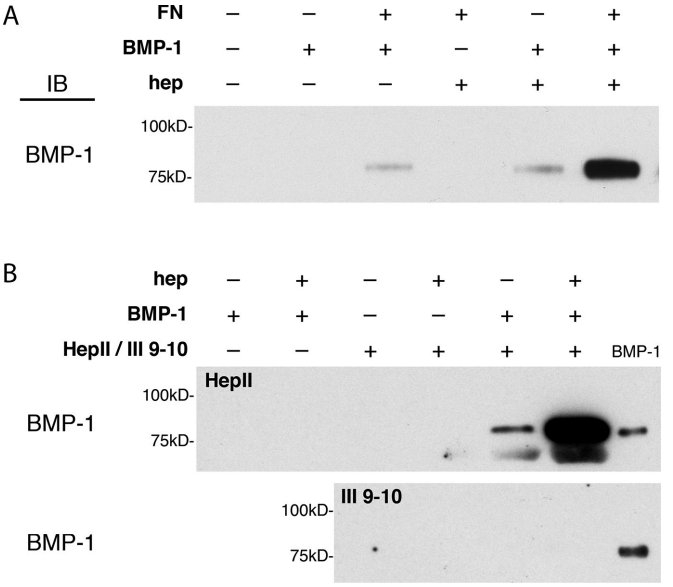

FIGURE 4: Heparin promotes BMP-1 binding to the HepII domain of FN. (A) FN in PBS (20 µg/ml) was mixed with 1 µg/ml rhBMP-1 (10:1 M ratio) ± 5 µg/ml heparin and incubated for 1 h. FN was pulled down using gelatin-Sepharose beads, and bound BMP-1 was detected by immunoblotting with anti–BMP-1 antibody. (B) Recombinant GST-tagged HepII domain of FN (1 µg/ml) was incubated with BMP-1 and heparin as in A. GST-III9-10 was used as a control. GST proteins were pulled down with glutathione beads and immunoblotted with anti–BMP-1 antibodies. Two nanograms of pure rhBMP-1 was electrophoresed in the right-hand lane. Data are representative of three (A) and four (B) experiments.

For examination of the direct effects of BMP-1 and heparin on processing, the time-course experiment was performed using a recombinant mini-procollagen (rhPro-COL1A1) in buffer as the substrate instead of procollagen in cell-conditioned medium. Again, cleavage of the C-propeptide took longer than 30 min with observable unprocessed rhPro-COL1A1 remaining at the conclusion of the experiment (Figure 5C). In the presence of heparin, cleavage by BMP-1 was completed in less than 30 min (Figure 5D). Control samples of conditioned medium or rhPro-COL1A1 with or without heparin showed no observable procollagen processing by the conclusion of the time course (Supplemental Figure S6, A–D). These results further support the conclusion that heparin enhances the rate of procollagen I processing by rhBMP-1 in solution. Because it binds to both procollagen and BMP-1, heparin may act as a scaffold to promote interactions and processing.

Procollagen processing by matrix-bound BMP-1

To determine whether BMP-1 localization to the ECM supports procollagen processing, we tested the ability of rhBMP-1 bound to an FN matrix to cleave rhPro-COL1A1. GM03349 fibroblasts were seeded at increasing densities to yield cultures with increasing

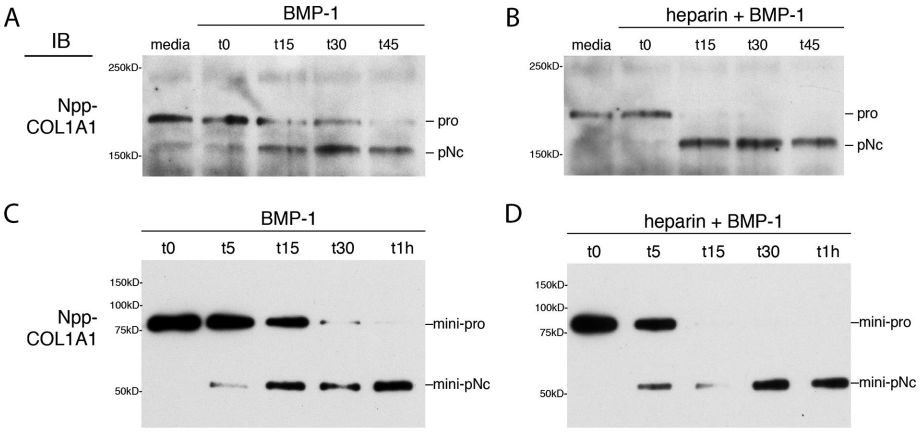

FIGURE 5: Heparin enhances procollagen cleavage by rhBMP-1 in solution. GM03349-conditioned medium (A, B) or rhPro-COL1A1 (C, D) was mixed with rhBMP-1 ± heparin and incubated for the indicated times. Aliquots were removed from each reaction and stopped with a solution of SDS, DTT, and EDTA. Time-course samples from reactions supplemented only with rhBMP-1 (A, C) or with rhBMP-1 plus heparin (B, D) were immunoblotted with anti-procollagen N-propeptide antibodies. Untreated GM03349-conditioned media were electrophoresed in the first lanes (A, B). Locations of procollagen (pro), pN collagen (pNc), or mini-procollagen (mini-pNc) equivalents are indicated to the right of each blot. Molecular mass markers are to the left of each panel. Blots are representative of four experiments. See Supplemental Figure S5A for quantification.

DISCUSSION

Procollagen proteolytic processing is required for nascent collagen fibrillogenesis, and in this study, we analyzed the role of FN matrix in procollagen cleavage by BMP-1, its C-propeptide proteinase. Both procollagen I and BMP-1 colocalize with FN matrix fibrils, and blockade of FN matrix assembly eliminated proteolytic processing of procollagen. BMP-1 binds to an FN-rich matrix in a dose-dependent manner and procollagen I was cleaved by matrix-bound BMP-1. We mapped a BMP-1–binding site to FN's HepII heparin-binding domain and showed that the addition of heparin increases the binding of BMP-1 to FN while also increasing procollagen cleavage by FN matrix-bound proteinase. Our results indicate that collagen fibrillogenesis depends on the FN matrix at least in part because FN fibrils provide binding sites for BMP-1 to facilitate procollagen processing.

Both BMP-1 and pNcollagen I colocalize with FN matrix fibrils, suggesting that the FN matrix acts as a scaffold that positions proteinase and substrate together to promote cleavage. BMP-1 has been shown to bind to FN at multiple sites (Huang *et al.*, 2009), which would allow a high density of proteinase molecules within the matrix and thus increase the chances that C-propeptide cleavage sites on FN-bound procollagen would be adjacent to FN-bound BMP-1. As heparin/HS is a known binding partner of both FN and BMP-1, it is possible that heparin acts as a bridge to link BMP-1 and FN. HS proteoglycans can exhibit scaffold-like behavior bringing multiple proteins into close proximity in their roles as structural components of the ECM, as receptors promoting ligand-receptor clustering, and as storage sites for growth factors and morphogens (Sarrazin *et al.*, 2011). In the case of an FN matrix, BMP-1 would not only bind directly to FN, but heparin binding to FN's HepII domain would add BMP-1–binding sites, because it is likely that each heparin chain (at ~70 saccharides) can bind to multiple BMP-1 molecules simultaneously. This type of linker role for heparin has recently been implicated in promoting FN matrix assembly by concomitant binding of a single heparin chain to multiple FN molecules (Raitman *et al.*, 2018).

amounts of FN matrix (Supplemental Figure S7). rhBMP-1 was added to the culture medium and allowed to bind to the matrix; unbound BMP-1 was removed; and cultures were then incubated with rhPro-COL1A1 for 3 h. Aliquots were immunoblotted using an anti–procollagen I N-propeptide antibody. No cleavage was detected with the addition of heparin without rhBMP-1 (Supplemental Figure S8). Samples treated with rhBMP-1 only show very little cleavage at the lowest cell densities but increasing rhPro-COL1A1 cleavage at higher FN matrix levels (Figure 6A). Quantification confirms that cleavage primarily occurs at the two highest cell densities (Supplemental Figure S5B). This experiment was repeated, but exogenous heparin was included during the incubation of rhBMP-1 with the matrix. Immunoblots of samples with rhBMP-1 plus heparin show an enhancement of rhPro-COL1A1 processing with detectable cleavage, fivefold higher than without heparin, at even the lowest cell density (Figure 6B and Supplemental Figure S5B). Taken together, these results show that, as with BMP-1 in solution, heparin also enhances procollagen I cleavage by matrix-bound BMP-1.

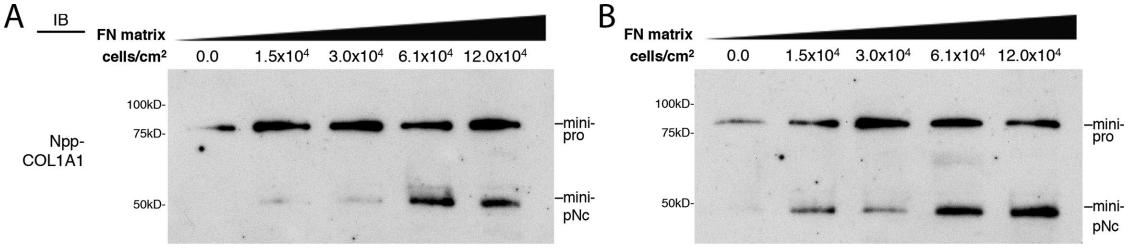

FIGURE 6: Heparin enhances processing of procollagen by matrix-bound BMP-1. GM03349 cells plated at various densities (cells/cm^2) were grown for 72 h to yield increasing amounts of FN matrix indicated by the black triangle (see Supplemental Figure S7). Cultures were then incubated with rhBMP-1 (A) or rhBMP-1 + heparin (B) followed by rhPro-COL1A1 for 3 h. The rhPro-COL1A1 solution was collected, and equal aliquots were immunoblotted with an anti-procollagen N-propeptide antibody for observation of rhPro-COL1A1 processing. Locations of full-length mini-procollagen (mini-pro) and C-propeptide-cleaved/ N-propeptide-retaining mini-procollagen (mini-pNc) are indicated (right). Blots are representative of four experiments. See Supplemental Figure S5B for quantification.

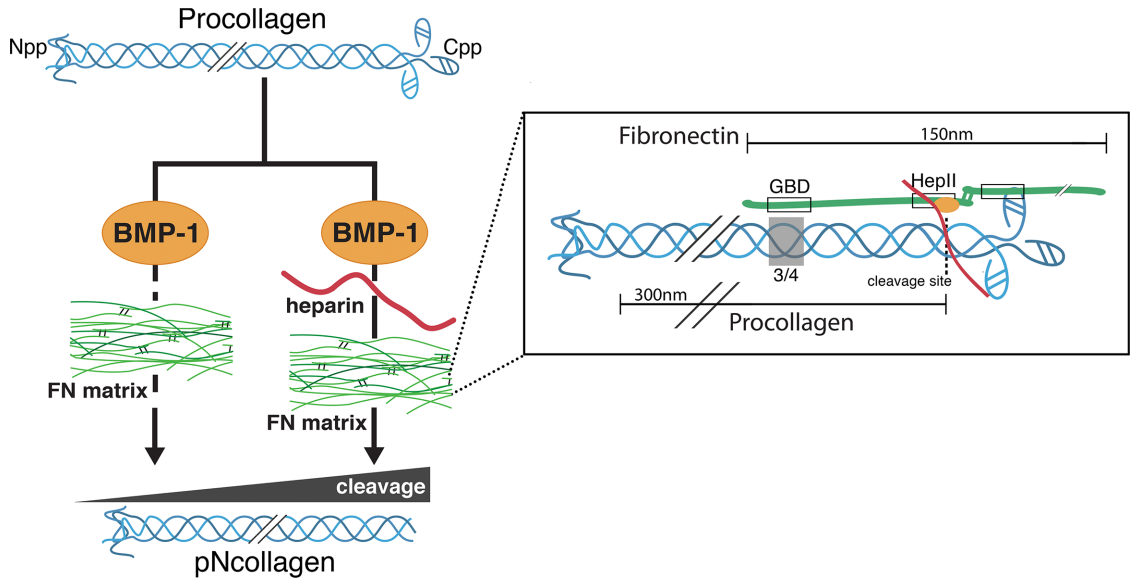

FIGURE 7: Model for FN-dependent proteolytic processing of procollagen I. Left, proteolytic cleavage of the C-terminal propeptide of procollagen I occurs when BMP-1 is bound to the FN matrix. An increased rate of processing is observed when both BMP-1 and heparin (or HS) are added together to the matrix. Right, example of procollagen and FN alignment to facilitate processing. Procollagen I contains a ¾ FN-binding site located ~225 nm (or ¾ of the distance) from the N-terminus of a fully processed, 300-nm-long collagen molecule (Dzamba *et al.*, 1993; Erat *et al.*, 2013). When this site is aligned with the collagen/gelatin-binding domain (GBD) on FN, the HepII domain of FN, located 55–60 nm from the N-terminus (Dzamba and Peters, 1991), is adjacent to the cleavage site of the C-propeptide of procollagen. Heparin binding to FN could facilitate procollagen cleavage by accumulating BMP-1 molecules at this site. Heparin/HS could also act as a scaffold for cleavage by simultaneously binding to BMP-1 molecules and to the C-terminal heparin-binding site on procollagen (San Antonio *et al.*, 1994).

In addition to FN and BMP-1, heparin/HS also binds to collagen. In fact, heparin enhances the rate of binding of FN to immobilized collagen (Johansson and Hook, 1980), and a ternary complex of heparin–FN–gelatin is more stable and requires higher concentrations of denaturant to dissociate than an FN–gelatin complex (Ruoslahti and Engvall, 1980). Considered together with our data, heparin/HS binding to FN may function to recruit both BMP-1 and procollagen, increasing their local concentrations to promote C-propeptide cleavage.

Another possible explanation of the effects of heparin is that its binding to FN induces a conformational change that may have an impact on the binding site for BMP-1. Conformational changes in FN upon heparin binding have been shown to expose binding sites for growth factors (Mitsi *et al.*, 2006, 2008; Li *et al.*, 2015; Vogel *et al.*, 2016). In at least one case, the association of heparin with FN was transient, but the cryptic binding sites remained available after heparin dissociation (Mitsi *et al.*, 2008). Therefore, it is possible that heparin binding to FN reveals a cryptic binding site for BMP-1 that remains even after heparin dissociation.

The effects of FN matrix plus heparin on BMP-1 processing of procollagen, combined with the fact that FN, BMP-1, heparin/HS, and procollagen are all binding partners, lead us to propose the following model for procollagen processing. In solution, BMP-1 is able to cleave the C-propeptide of procollagen I, but the addition of heparin increases the rate of that reaction. In this situation, heparin could be acting as a scaffold, providing binding sites for multiple BMP-1 and procollagen molecules to bring them into close proximity for cleavage. In the matrix microenvironment in vivo, FN matrix-bound BMP-1 cleavage of procollagen is also enhanced by the addition of heparin (Figure 7). In this case, the FN matrix is the scaffold. Heparin could be tethering more BMP-1 and procollagen molecules to FN fibrils or it could be changing the existing BMP-1–binding sites through conformational changes in FN. Our results do not distinguish between these mechanisms and, in fact, these mechanisms are not mutually exclusive. In addition to its interactions with BMP-1, heparin might also have a procollagen-specific role by stabilizing its interactions with FN during processing. For example, procollagen binding to FN's collagen/gelatin-binding domain via its ¾ site could position FN's HepII domain with its BMP-1–binding site adjacent to the BMP-1 cleavage site (Figure 7). This localization hypothesis is supported by the reported locations of the ¾ FN-binding site along the 300-nm collagen I molecule (Miller and Gay, 1987; Canty and Kadler, 2005; Wess, 2005), and the collagen/gelatin-binding domain on FN (Singh *et al.*, 2010). The distance from the procollagen C-propeptide cleavage site (Greenspan, 2005) to collagen's ¾ binding site allows for BMP-1 bound to a HepII domain of the FN dimer to act upon procollagen that is tethered to FN at its collagen/gelatin-binding domain. BMP-1's enhancer protein, PCPE-1, which has been shown to bind FN (Weiss *et al.*, 2014), may also have a part in this mechanism. We also did not detect N-propeptide cleavage when FN matrix assembly was blocked, raising the possibility that enzymes involved in N-terminal processing might also be affected by the ECM. It appears that multimolecular interactions with a scaffolding structure, either FN matrix in vivo or heparin in solution, control the rate of procollagen proteolytic processing and would thus provide several different mechanisms to control collagen fibrillogenesis.

MATERIALS AND METHODS

Cells and reagents

Human primary dermal fibroblasts (GM03349; Coriell Institute, Camden, NJ) were grown in MEM containing 1% nonessential amino acids, and 1 mM sodium pyruvate (all from Life Technologies, Grand Island, NY), supplemented with 15% fetal bovine serum

(Hyclone, Logan, UT) and antibiotic/antimycotic cocktail (Corning Life Sciences, Oneonta, NY). Cells were tested and found to be free of mycoplasmal contamination. For inhibition of FN matrix assembly, cells were seeded in medium containing the bacterial peptide FUD or a control peptide III-11C at 2.4 μg/ml and grown for 5 d (Maurer *et al.*, 2010; Hunt *et al.*, 2012). FUD and III-11C were purified as described previously (Tomasini-Johansson *et al.*, 2001).

Fibronectin was purified from fresh frozen human plasma by gelatin-Sepharose affinity chromatography (Wilson and Schwarzbauer, 1992). GST fusion proteins were expressed and purified as described previously (Williams and Schwarzbauer, 2009). Heparin from porcine intestinal mucosa (Grade I-A, ≥180 USP U/mg) was obtained from Sigma (St. Louis, MO). Recombinant human BMP-1 (rhBMP-1) and recombinant human mini-procollagen (rhPro-COL1A1) were obtained from R&D Systems (Minneapolis, MN). According to the manufacturer, rhPro-COL1A1 is resistant to pepsin digestion, indicating triple helical structure, and is cleaved by rhADAMTS-3, which only cleaves native collagen.

Antibodies used in this study include R184 rabbit anti-FN antiserum raised in-house against the rat FN $III_{1\text{-}6}$ domain; hFN7.1 anti-human FN monoclonal antibody that recognizes FN $III_{9\text{-}10}$ repeats (supernatant; DSHB, Iowa City, IA); PA2140-2 rabbit anti–collagen I polyclonal antibody against the C-terminus of mouse type I collagen (Boster Biological Technology, Pleasanton, CA); SP1.D8 anti–procollagen I antiserum against the N-terminal propeptide (supernatant; DSHB, Iowa City, IA); 3E9 monoclonal anti–BMP-1 antibody (OTI3E9; OriGene Technologies, Rockville, MD); and 6x-His Tag monoclonal antibody (3D5; Thermo Fisher Scientific). Alexa Fluor–conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were from Invitrogen (Eugene, OR).

Immunoblotting

Cells were lysed in buffered DOC; this was followed by separation into DOC-soluble and DOC-insoluble fractions by centrifugation (Sechler *et al.*, 1996). Insoluble material was solubilized in 2% SDS buffer. In some experiments, whole-cell lysates were prepared by lysis in 4% SDS lysis buffer. Proteins were separated by SDS–PAGE on 5 or 8% polyacrylamide gels alongside Precision Plus Protein Standard (Bio-Rad Laboratories, Hercules, CA) and then transferred to nitrocellulose membrane (GE Healthcare Life Sciences, Marlborough, MA). Incubations were performed as previously described (Sechler *et al.*, 1996) with the following antibodies: R184 (1:50,000), 3E9 (1:8000), PA2140-2 (0.15 μg/ml), and SP1.D8 (0.2 μg/ml). Secondary antibody was either horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG) or horseradish peroxidase–conjugated goat anti-mouse IgG diluted 1:50,000 (Pierce Chemical, Rockford, IL). Blots were developed using Super-Signal West Pico ECL reagents (Pierce Chemical). Densitometry was performed on scanned films using Fiji (Schindelin *et al.*, 2012), and exposures yielding signals within the linear range were quantified.

Modified ELISA and pull-down assay

Cells were seeded and grown for 72 h to confluence in a 96-well plate with 50 μg/ml ascorbic acid (Sigma, St. Louis, MO). Cells were washed and incubated with buffer, 0.5 μg/ml, 1.0 μg/ml, 5.0 μg/ml, or 10.0 μg/ml rhBMP-1 in phosphate-buffered saline (PBS) with 0.2 mg/ml bovine serum albumin (BSA). For preparation of a cell-free matrix, cells were grown past confluence in a 96-well plate with 50 μg/ml ascorbic acid, and then cultures were decellularized to remove the cellular material and leave behind the fibrillar FN matrix as previously described with the addition of a 20 U/ml DNase I (New England Biolabs, Ipswich, MA) incubation (Harris *et al.*, 2018). Decellularized matrices were incubated with PBS plus 0.2 mg/ml BSA with or without rhBMP-1 at 1.0 or 5.0 μg/ml. BMP-1 binding was detected with 3E9 anti–BMP-1 antibody (1:100), and the presence of FN was confirmed with hFN7.1 anti-FN antibody. Antibodies were detected by incubation with biotinylated goat anti-mouse IgG and streptavidin-β-galactosidase. Substrate pNPG (*p*-nitrophenyl β-D-galactopyranoside) was then added to initiate colorimetric readout and stopped with 0.5 M sodium carbonate. Optical density at 405 nm was determined using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA).

For analysis of binding of BMP-1 to FN, BSA-blocked gelatin–Sepharose beads (GE Healthcare Life Sciences, Marlborough, MA) were incubated with 20.0 μg/ml FN and 1.0 μg/ml rhBMP-1 with or without addition of 5.0 μg/ml heparin in PBS at 4°C for 1 h with rotation. FN plus bound components was collected by centrifugation. Proteins were eluted with electrophoresis sample buffer and analyzed by SDS–PAGE and immunoblotting. A similar procedure was used to analyze BMP-1 binding to FN domains GST-HepII and GST-III9-10, except pull down was accomplished using glutathione Sepharose 4B beads (GE Healthcare Life Sciences).

Immunofluorescence staining and microscopy

For immunostaining, cells were washed with PBS and fixed in 3.7% formaldehyde in PBS. For BMP-1 localization, cells were incubated with 1.0 μg/ml rhBMP1 or equivalent volume of buffer in medium without serum, containing 5.0 μg/ml BSA, for 30 min at 37°C, and then washed and fixed for immunostaining. Primary antibodies were used at the following dilutions: R184 at 1:100, SP1.D8 at 1:20, hFN7.1 at 1:10, PA2140-2 at 1.0 μg/ml, 3E9 at 1:100, anti-6xHis at 1:500, and Alexa Fluor secondary antibodies at 1:500. All were diluted in 2% ovalbumin in PBS. Coverslips were mounted using ProLong Gold Antifade Reagent (Life Technologies, Grand Island, NY). Images were captured using a Nikon Eclipse Ti microscope equipped with a Hamamatsu C10600 ORCA-R2 digital camera and iVision image acquisition software, or a Nikon A1 confocal microscope with Nikon Elements image acquisition software. Images were equivalently adjusted using Fiji software.

Procollagen processing assays

For procollagen I cleavage, GM03349-conditioned medium was supplemented with 0.5 μg/ml rhBMP-1 ± 5.0 μg/ml heparin. For rhPro-COL1A1 time courses, rhPro-COL1A1 in dilution buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$) was mixed with rhBMP-1 in assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 150 mM NaCl) in a 1:3 ratio, as per the manufacturer's instructions (R&D Systems, Minneapolis, MN). Final concentrations were 18.75 μg/ml rhPro-COL1A1, 3.75 μg/ml rhBMP-1, 25 mM Tris-HCl, 2.5 mM $CaCl_2$, and 112.8 mM NaCl, supplemented with 5.0 μg/ml heparin. Titration of heparin was used to determine an appropriate concentration for these experiments (Supplemental Figure S4B). Reactions were incubated at 37°C for up to 1 h. Aliquots were removed from each reaction at 15-min intervals, and the reaction was stopped by addition of SDS, dithiothreitol (DTT), and EDTA to final concentrations of 2% SDS, 0.1 M DTT, and 7 mM EDTA.

Cells were seeded at various densities in a 96-well plate and grown for 72 h (Supplemental Figure S7). Cells were washed and incubated with ±1.0 μg/ml rhBMP-1 ± 5.0 μg/ml heparin in 1 mg/ml BSA in PBS for 30 min at 37°C. Unbound BMP-1 and heparin were removed, and 0.25 μg/ml rhPro-COL1A1 in buffer containing 50 mM Tris-HCl (pH 7.5) and 5 mM $CaCl_2$ was added and incubated for 3 h at 37°C. rhPro-COL1A1 solution was removed, and the reaction was stopped as above.

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