FIBRONECTIN MATRIX AS A SCAFFOLD FOR PROCOLLAGEN PROTEASE BINDING AND COLLAGEN PROCESSING

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ABSTRACT

The extracellular matrix (ECM) proteins fibronectin (FN) and type I collagen (collagen I) are co-distributed in many tissues and collagens have been shown to depend on a FN matrix for fibrillogenesis. Despite extensive understanding of supramolecular collagen fiber structure and matrix deposition, the molecular interactions of the proteolytic processing of collagen’s biosynthetic precursor, type I procollagen (procollagen I), have not been elucidated, in particular the role of FN matrix in that process. Microscopic analysis of a fibroblast ECM showed co-localization of procollagen I with FN fibrils and inhibition of FN matrix assembly led to a significant reduction of proteolytic cleavage of procollagen to initiate fibril formation, and a concurrent enrichment of collagens containing one or both propeptides. We examined the role of FN matrix in procollagen processing by the C-propeptide proteinase BMP-1. We found that BMP-1, like procollagen, co-localizes 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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DEDICATION

This thesis is dedicated to my Uncle Lenny, who loves the autoclave for some reason.
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My time at Princeton in the Molecular Biology Department has been a wild ride, but it has been an experience that I would not trade for anything.

Since the very beginning, Elena Chiarchiaro offered me her support and would lend me an ear whenever I needed one. Melissa DiMeglio has been a real trooper over the last few weeks dealing with the bureaucracy behind an FPO and handling all of my frantic emails.

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ABBREVIATIONS

BMP-1 – Bone morphogenetic protein – 1
DOC – Deoxycholate
ECM – Extracellular Matrix
FN – Fibronectin
GAG – Glycosaminoglycan
GST – Glutathione S-transferase
HS – Heparan sulfate
HSPG – Heparan sulfate proteoglycan
pCcol – Collagen retaining the C-propeptide
PCPE-1 – Procollagen C-endopeptidase enhancer
pNcol – Collagen retaining the N-propeptide
CHAPTER 1

Introduction
Chapter 1.1 – Introduction to the Extracellular Matrix

The extracellular matrix (ECM) is a complex network of molecules that are secreted and assembled by cells (Figure 1.1-1). This network is integral for providing organization for cells, and structure and stability for surrounding tissues (Mecham, 2011). Cellular processes such as wound healing, differentiation, cell polarity establishment, and tissue regeneration all rely on a proper functional ECM (Hynes and Yamada, 2012).

The ECM is an incredibly dynamic structure that can adapt and remodel in response to cellular cues. Growth factors and other signaling proteins mediate this development of an ECM capable of maintaining homeostasis. The major components of the ECM—fibrous proteins, such as fibronectin (FN) and collagen, and proteoglycans interact to confer the necessary biomechanical properties of a matrix. Resilience to tensile and compressive forces, rigidity and elasticity, and porosity to allow for cell motility and selective filtration are imparted to the ECM through these interactions (Mecham, 2011).

Dysregulation or loss of any of these critical components can lead to a disease state. Abnormal accumulation of matrix proteins can result in fibrosis, scarring, or tumorigenesis, all of which increase ECM stiffness and result in loss of tissue function (Frantz et al., 2010). Additionally, chronic injury or prolonged exposure to toxins can lead to scarring (Mann et al., 2011). Many developmental processes rely on a proper ECM as well. Osteogenesis imperfecta (OI), otherwise known as brittle bone disease, results from improper development of connective tissue, where collagen is not processed properly (Forlino and Marini, 2016). This leads to abnormal collagen fibril assembly which produces fragile bones prone to breakage. Thus, accurate processing of matrix proteins, not just proper assembly, is immensely important for ECM functionality.
Figure 1.1-1 – The extracellular matrix (ECM). The ECM a complex, highly dynamic, network of fibrillar proteins, proteoglycans, glycoproteins, growth factors, and more. Fibronectin (FN), the ubiquitous fibrillar matrix protein, and type I collagen, a rigid fibrillar matrix protein are important to emphasize in the context of this thesis. FN (green) and type I collagen (blue), as well as collagen’s precursor procollagen (blue, propeptides on either terminus), bind to cell surface receptors called integrins (yellow) that play a role in fibrillogenesis and connecting the ECM to the cytoskeleton. Heparan sulfate proteoglycans (HSPGs, red) are present both in the extracellular space and span the cell membrane. Heparin, a highly sulfated analog to heparan sulfate (HS), can exist in the ECM as an independent glycosaminoglycan after cleavage from its protein core. HSPGs and heparin are known to interact with both FN and type I collagen, as well as with many other matrix proteins, enzymes (such as BMP-1), and growth factors not depicted here.
The mechanisms and regulation of collagen processing in the ECM are not well understood. With collagen being the most abundant ECM protein, an understanding of how a proper collagen matrix is assembled is necessary. The deposition of a collagen matrix is known to rely on the presence of a previously established FN matrix (McDonald et al., 1982). Therefore, a fundamental comprehension of how FN matrix regulates collagen processing and fibrillogenesis is essential to understand how a proper functional ECM is assembled.

Chapter 1.2 – Fibronectin Assembly Mechanisms

The assembly of an insoluble FN matrix from compact, dimeric molecules is an elegant and complex process that ensures a proper foundation for the future ECM and the functioning of an organism as a whole. Regular FN matrix assembly is essential for avoiding aberrant developmental processes. Mice that do not express the FN gene are embryonic lethal, presenting with shortened anterior-posterior axes, deformed neural tubes, an absent notochord and somites, and deformed embryonic vessels and heart (George et al., 1993). FN mutations in humans are rare, although heterozygous mutations have been observed to lead to abnormal skeletal development and the generation of spondylometaphyseal dysplasia (Cadoff et al., 2018).

The process of proper FN matrix assembly begins with the secretion of a dimeric FN molecule into the extracellular space (Schwarzbauer and DeSimone, 2011). The dimer is held together by a pair of disulfide bonds at the C-terminus of each monomer, which is quite large on its own, having a molecular weight of 230-270 kDa (Mao and
Schwarzbauer, 2005). Dimerization is necessary for matrix formation, as secreted monomeric FN cannot take part in matrix development (Schwarzbauer, 1991; Kozaki et al., 2003). The FN subunits are composed of 3 different repeating modular units, termed type I, II, and III repeats (Figure 1.2-1), that have distinct structures and roles in the mechanism of FN assembly (Xu and Mosher, 2011). The 15 type III repeats are distinct from the 12 type I and 2 type II repeats in that they lack intramolecular disulfide bonds to assist in stabilizing its folded structure. Type I repeats are comprised of stacked β-sheets, while type II repeats are two anti-parallel β-sheets. The type III repeats are seven-stranded β-barrels arranged in two anti-parallel β-sheets lacking any disulfide bonds.

The arrangement of these modular units into domains is important for the assembly of a FN matrix. Interactions between the FN molecule and αβ heterodimeric, transmembrane cell surface integrin receptors, mainly α5β1 integrin, are required for initiation of FN matrix assembly (which will be detailed further in the following paragraph) (Hynes, 1990). Integrins bind to FN through its III9-10 domain, composed of the III9 synergy site that allows for maximum integrin-FN association, and the III10 RGD site that is absolutely required for α5β1 integrin interaction (Schwarzbauer and DeSimone, 2011). Further N-terminal, repeats I1-5II1-2I7-9 comprise the 70 kDa fragment, a domain that consists of two other important sub-domains with respect to matrix assembly. The five most N-terminal type I repeats (I1-5) are necessary for FN assembly and are considered the major FN binding domain (Schwarzbauer, 1991). Recombinant FN that lacks part or all of this domain is not able to form fibrils. Repeats III1-2 and III4-5 are also important for FN fibrillogenesis (Sechler et al., 1996). Repeats I4II1-2I7-9 are the collagen/gelatin binding domain. This domain is known to bind to both collagen and its denatured
Figure 1.2-1 – Fibronectin (FN) is a multivalent fibrillar ECM protein. FN is ubiquitous ECM protein that forms a foundational matrix for many other ECM proteins to be deposited on. It exists as a dimer with a $M_w$ of approximately 500kDa, with each monomeric subunit having a $M_w$ of 250-270kDa. The subunits are composed of repeating domain structures designated as type I, II, and III repeats. The first 9 type I repeats, interrupted by the 2 type II repeats, comprise the 70kDa fragment. The assembly domain (repeats I1-I5 of the 70kDa fragment) is necessary for FN-FN self-association interactions, while I6 II1-II2 I7-I9 make up the collagen/ gelatin binding domain that associates with collagen. Within III9-II10 is the RGD site, which is where FN associates with integrins. The HepII domain is comprised of III12-II14 and is the major heparin-binding domain of FN. When inclusive of III15 and the entirety of the variable region between III14 and III15, the domain is referred to as HV120. We refer to HV120 as the HepII domain in this thesis.
Figure 1.2-2 – Assembly of an insoluble FN matrix is a step-wise process. (A) A compact FN dimer binds to integrins (α5β1). (B) Cytoskeletal connections between the cytoplasmic domain of integrins and actin lead to increased cell contractility and conformational changes in FN. This exposes cryptic binding sites on FN that allow for FN-FN self-association. (C) Integrins begin to cluster and FN-FN interactions occur. Further changes in conformation follow. (D) More FN-FN binding occurs, leading to the formation of a stable, insoluble matrix. The inset box shows end-to-end FN associations between N-terminal assembly domains, and lateral associations between other FN binding sites located in III1-2, III4-5, and III12-14. Adapted from Singh, Carraher, & Schwarzbauer, 2010.
form, gelatin, with a higher affinity for gelatin (Engvall et al., 1978). This domain is crucial for collagen matrix assembly, as recombinant FN that lacks this domain, or in cell cultures where access to this domain is blocked, no collagen matrix is deposited (Sottile et al., 2007; Shi et al., 2010). Additionally, FN contains multiple heparin-binding domains (HepI: I_1-6, HepII: III_12-14, HepIII: III_3-5) (Hynes, 1990). The HepII domain is the major heparin binding site on FN (Barkalow and Schwarzbauer, 1991).

Access to many of these domains relies on FN fibrillogenesis and matrix assembly after FN is secreted as a compact dimer from the cell (Figure 1.2-3). Once FN is integrin-bound, intracellular proteins, such as talin and paxilin, are recruited to the cytoplasmic domain of the integrin and connect to the actin cytoskeleton (Xu and Mosher, 2011). Contractility of the actin cytoskeleton induces conformational changes in the FN, revealing cryptic FN-FN binding sites. Integrin clustering occurs, and the newly accessible FN-FN self-association sites are able to interact between adjacent FN molecules, leading to fibrils. Soon, stable, insoluble fibrils form as more FN dimers with open conformations interact end-to-end with other FN molecules via their N-termini, or through lateral associations. This eventually leads to the formation of a stable, foundational FN extracellular matrix with fully accessible domains for further ECM interactions.

Chapter 1.3 – The Collagens as a Major Matrix Molecule– Synthesis, Assembly, and Matrix Deposition

Collagens are a large, heterogeneous family of proteins that comprise the majority of the ECM and are the most abundant protein in the vertebrate body (Ricard-Blum,
2011). In fact, one-third of the total protein in the human body is collagenous (Shoulders and Raines, 2009). As of now, the collagen family contains 28 distinct types of collagen (type I-XXVIII) that are coded for by at least 41 different genes, to create at least 46 different polypeptides (Brinckmann, 2005). These diverse proteins impart numerous important characteristics on the ECM, ranging from tensile strength, stability, and rigidity. This is accomplished through the fabrication of various arrays of collagen molecules, as is displayed by the fibrillar collagens type I-III, V, and XI (Birk, 2005). These collagens can form supramolecular assemblies called fibrils that are crucial for proper development and maintenance of the human body. Packed, parallel bundles of fibers create tensile tendons, lateral arrays of fibers create the transparent cornea, and concentric, weaved fibers result in rigid, stable bone (Maurice, 1957; Birk and Trelstad, 1986; Canty and Kadler, 2005). Upregulation of type I collagen occurs during precartilage mesenchymal cell condensation for chondrogenesis, or cartilage formation, and type II and IX (a fibril-associated collagen) collagen facilitate chondrocyte proliferation and differentiation (Singh and Schwarzbauer, 2012). Missteps in these collagen-based processes result in varied disease states. Ehlers-Danlos syndrome is a direct result of erroneous extracellular type I collagen processing (Cabral et al., 2005), while absence of the intracellular type I collagen chaperone protein Hsp47 results in improper collagen folding and retention in the endoplasmic reticulum, leading to embryonic lethality (Nagai et al., 2000; Ishida et al., 2006). As our understanding of the regulatory elements of these collagen-based processes and their associated pathologies grow, development of impactful therapies for collagen-based diseases can emerge.
Figure 1.2-3 – A FN matrix is fibrillar and dense. Human lung fibroblasts (WI38) were grown for 10 days and supplemented with 50.0μg/mL ascorbic acid every 2 days. Cultures were decellularized by applying buffers containing detergents at varying pH levels. Decellularized cultures were immunostained using hFN 7.1, a monoclonal FN antibody against III_{9–10}, and the appropriate Alexa Fluor secondary antibody. Image was acquired at 20x magnification, with FN represented in white.
The diverse suprastructures found in the collagen family may appear complex and incredibly varied, but they do require certain baseline architecture. Each collagen molecule is composed of three polypeptides, or α chains, leading to the formation of either heterotrimers or homotrimers (Hulmes, 2008). The α chains have a repeating glycine-X-Y structure, with X commonly being proline and Y being hydroxyproline. The triple helical structure is characteristic of collagens, but does not necessarily comprise a majority of the molecule as a whole. For the major fibrillar collagens (type I-III, V, XI) this hallmark triple helix can comprise up to 96% of the molecule, while the type XII collagen structure is made up of less than 10% of this triple helix (Ricard-Blum, 2011). This discrepancy in structure divides the collagen family into distinct subgroups: fibrillar, transmembrane, network, FACIT (fibril associated collagens with interrupted triple helices), and MULTIPLEXIN (multiple triple helix and interruptions). The FACITs (types IX, XII, XIV, XVI, XIX, XX, XXI, and XXII) do not form fibrils, but rather associate with the surface of collagen fibrils. This is in contrast to the network collagens (types IV, VIII, and X) that can form hexameric or hexagonal networks, and transmembrane collagens (types XIII, XVII, XXIII, and XXV) that can behave as cell-surface receptors or as matrix components if they are shed by the cell through proteolysis (Franzke et al., 2005).

For the fibrillar collagens, particularly type I collagen, synthesis and assembly are stepwise and tightly regulated. After synthesis of the α chains in the rough endoplasmic reticulum, trimerization is directed in a zipper-like fashion starting at the trimerization, or oligomerization, domain (Engel and Prockop, 1991). This C-terminal domain directs interconnection of the C-terminal propeptide that will lead to a non-helical domain, called
the telopeptide, which flanks the triple helical portion of the molecule on either termini before the N-terminal propeptide is assembled (Hulmes, 2002). This newly fabricated molecule is considered a biosynthetic precursor to collagen, called procollagen (Figure 1.3-1A). This precursor molecule is highly soluble compared to collagen; a property determined by the presence of the globular N-terminal propeptide and more so the pseudo-cruciform structured C-propeptide (Bernocco et al., 2001).

After procollagen is secreted, the propeptides are proteolytically cleaved to form a mature collagen molecule that can form arrays. For type I collagen, the N-propeptide is cleaved by ADAMTS-2 (a disintegrin and metalloproteinase with thrombospondin motifs) and the C-propeptide is cleave by BMP-1 (bone morphogenetic protein), leaving a fully processed type I collagen molecule that is ~300nm long and ~1.5nm in diameter (Miller and Gay, 1987; Birk, 2005). This stepwise processing can be observed biochemically through immunoblots of conditioned media from cell cultures, presenting as a series of three bands subsequently decreasing in molecular weight (Figure 1.3-1B).

Once processing has occurred, fibrillogenesis can begin through a self-assembly process whereby collagen molecules associate with one another and are stabilized through intermolecular cross-linking. Type I collagen fibrils have been well characterized, having been observed to range in diameter from ~15nm to 500nm or more, and present with a distinctive banding pattern with a periodicity (D) of 64-67 nanometers (Birk and Linsenmayer, 1994).

The self-assembly of collagen molecules can occur in solution in the absence of FN, however, matrix deposition relies on an established FN matrix (Dzamba and Peters, 1991; Velling et al., 2002) (Figure 1.3-2) with accessible collagen/ gelatin binding
Figure 1.3-1 – Collagen is proteolytically processed from its biosynthetic precursor procollagen. A) Procollagen is the soluble precursor molecule of collagen. It is comprised of the conventional triple helical domain flanked on either terminus by short non-helical domains called telopeptides. Together, these form the 300nm long rod-like molecule of collagen. Procollagen has propeptides on both termini, called the N- and C-propeptides. The N-propeptide is globular, while the C-propeptide adopts a pseudo-cruciform structure. These propeptides are cleaved by two distinct proteinases, ADAMTS-2 and BMP-1, for the N-propeptide and C-propeptide, respectively. One of the propeptides of procollagen is cleaved in the initial processing step. Normally, the C-propeptide is cleaved first due to the necessity of its removal for fibrillogenesis. There is evidence of N-propeptide processing occurring first, but often this is due to disorders that affect C-propeptide processing. Once both propeptides are cleaved, a fully processed collagen molecule is produced. B) Processing of procollagen can be observed through immunoblotting. WI38 human lung fibroblast conditioned media was immunoblotted with an anti-type I collagen antibody. (1) Unprocessed procollagen at ~200kDa. (2) Cleavage of a single propeptide has occurred. Slight differences in M<sub>w</sub> between the two propeptides create a doublet at ~150kDa. (3) A band with a M<sub>w</sub> of 100-125kDa appears once both propeptides are cleaved and fully processed collagen is present. (4) As processing continues, only collagen retaining a single propeptide (pNcollagen or pCcollagen) remains along with the fully processed collagen.
domains (Balian et al., 1980; Erat et al., 2013). Recombinant FN that is lacking the collagen / gelatin binding domain can form a matrix, but collagen fibril deposition is inhibited (Sottile et al., 2007). Similarly, cell cultures treated with the bacterial peptide R1R2, which binds specifically to the collagen / gelatin binding domain on FN and blocks access, had inhibited collagen matrix assembly. This reliance of collagen on the FN matrix is emphasized by FN and type I collagen co-localizing in ECM (Figure 1.3-3). Moreover, collagen matrix turnover is regulated by the rate of FN matrix turnover (Shi et al., 2010).

FN binding sites on collagen must be accessible as well. Collagen contains two FN binding sites: the 1/10 site and the 3/4 site, named after their location along the 300nm collagen molecule (Erat et al., 2009). The 1/10 site begins ~30nm in from the N-terminus of fully processed collagen. Thus, the 3/4 site begins ~75nm in from the N-terminus of a fully processed collagen molecule. These FN binding sites are crucial for FN interactions that regulate collagen matrix assembly, and interestingly, there is evidence that the collagen matrix can regulate fibronectin fibrillogenesis as well through these same interactions. Fibroblasts that do not express endogenous α1(I) collagen chains were shown to produce a sparse matrix comprised of short FN fibrils (Dzamba et al., 1993). Transfection with the pro-α1(I) gene rescued FN matrix formation. Furthermore, introducing mutations into the FN binding region of the pro-α1(I) gene led to a sparse FN matrix that was only rescued upon addition of type I collagen. Essentially, just as the establishment of FN matrix is necessary for the deposition of a collagen matrix, the presence of soluble procollagen / collagen is necessary for the formation of a FN matrix.
Figure 1.3-2 – Collagen forms a fibrillar matrix. Human dermal fibroblasts (GM03349) were grown to confluence and supplemented with 50.0μg/mL ascorbic acid to promote collagen synthesis. Cultures were immunostained using an anti-type I collagen antibody and the appropriate Alex Fluor secondary antibody. Image was acquired at 40x magnification, with collagen represented in white.
Figure 1.3-3 – FN and collagen co-localize in the ECM. GM03349 cells were grown to confluence and co-immunostained using an anti-type I collagen and anti-FN antibody, along with the appropriate Alexa Fluor secondary antibodies. Images were acquired using a Nikon A1 confocal microscope with 40x magnification. Collagen is represented in green and FN is represented in red. Yellow indicates regions of co-localization.
Type I collagen, its importance in the ECM and the human body, its intracellular synthesis, post-translational modifications, and self-assembly into supramolecular structures capable of matrix formation are well-documented in the literature. Despite the extensive knowledge base on collagen synthesis, processing, assembly, and regulation that has been curated, there are still unknowns about collagen that remain. The proteolytic processing of the propeptides of newly-secreted procollagen and the initiation of fibrillogenesis for the fully processed collagens are not as detailed. The question still remains: What regulates procollagen processing and nascent collagen fibrillogenesis?

**Chapter 1.4 – Matrix Metalloproteinases and the Procollagen-Specific BMP-1 Proteinase**

Matrix metalloproteinases (MMPs) are a large family of endopeptidases that can act upon specific peptide bonds of ECM proteins, or degrade the entire protein itself. MMPs share the structural feature of an active site Zn$^{2+}$, imparting the metalloproteinase name, and contain the two conserved motifs of a pro-domain and catalytic domain (Gill, 2011). When the pro-domain is present, the proMMP is kept in a latent state through an interaction between a zinc ion and a cysteine thiol present in the prodomain consensus sequence PRCXXPD. Catalytic activity of the MMP can only be gained once this interaction is disrupted. Regulation of MMP activation is only understood for a subset of MMPs that contain a furin recognition sequence between the pro- and catalytic domains which is acted upon by furin intracellularly to activate the enzyme (Leighton and Kadler, 2003). This regulation is paramount, as unregulated MMP activity can lead to disease states that include fibrosis, inflammation, Alzheimer’s disease, and multiple sclerosis.
Interestingly, many MMP-null mouse models did not present with any directly related developmental phenotypes. However, knocking out MMPs did affect collagen turnover, causing altered growth plate development and vascularization, arthritis, and dwarfism (Vu et al., 1998; Holmbeck et al., 1999; Inada et al., 2004).

In mice and humans, the astacin family of proteases is a sub-group of specialized MMPs that are secreted by the cell and act on or near the cell surface (Norman et al., 2003). This family includes the MMPs TLL1, TLL2, MEP1α, MEP1β, ASTL, and most notably BMP-1 (Stöcker and Gomis-Rüth, 2013). ECM degradation is one of the diverse biological processes in which this family of proteases participates, including early embryo morphogenesis and tissue differentiation (Gomis-Ruth, 2003). This makes tissue-specific expression in developing organisms immensely important for the astacins. However, since no endogenous inhibitors for astacins have been discovered, except for the broad-spectrum plasma protease inhibitor α2-macroglobulin (Zhang et al., 2006), erroneous activity goes unchecked.

BMP-1 (bone morphogenetic protein 1), a member of the astacin MMPs, is incredibly important in the processing of the fibrillar collagen, type I collagen. Despite its name, BMP-1 is not a part of the TGFβ growth factor family of morphogens. It was discovered in bone extracts and was observed to be capable of inducing bone formation (Wozney et al., 1988), prompting the misleading name. Later, a splice variant for this zinc-dependent MMP was discovered, and termed mammalian tolloid (mTLD) (Takahara et al., 1994). BMP-1 cleaves the C-propeptide of type I procollagen, which is necessary for assembly of collagen I molecules and fibrillogenesis (Hopkins et al., 2007). In contrast, collagen I fibrils that retain the N-propeptide (pNcollagen) have been observed
in vivo, and fibrillar assemblies of \( \text{pNcollagen I} \) can be formed in vitro from purified protein (Prockop and Hulmes, 1994). Developmentally, BMP-1 is required for ventral body wall closure, and BMP-1 null mice have been observed to be perinatal lethal with malformed collagen fibrils and lacking the fold that encloses the gut (Suzuki et al., 1996).

Structurally, BMP-1 is comprised of an N-terminal pro-domain, which is cleaved by furin-like / paired basic proprotein convertases in the trans-Golgi (Figure 1.4-1A). Cell cultures treated with furin inhibitors presented with secretion of pro-BMP-1 that could not process procollagen (Leighton and Kadler, 2003). This was rescued by the addition of recombinant furin, and showed that cleavage of the pro-domain is not required for secretion. Similar results were obtained when TGF-\( \beta \)1 induced pro-BMP-1 and BMP-1 secretion (Lee et al., 1997). Following the N-terminal pro-domain is an astacin-like catalytic domain, followed by three CUB (complement C1r/C1s, \( \mu \)EGF, BMP-1; CUB1, CUB2, CUB3) domains and an EGF (epidermal growth factor-like; EGF1) domain (Greenspan, 2005). The mTLD splice variant is actually larger, and contains five CUB domains and two EGF domains. Later, a study revealed that only the metalloproteinase / astacin-like catalytic domain and the CUB2 domain are required for BMP-1 activity on type I procollagen (Petropoulou et al., 2005).

Through the application of small-angle x-ray scattering (SAXS), a BMP-1 structure was developed. The proposed structure has a horseshoe-like shape where a cleft is formed that contains the catalytic domain and the catalytic zinc ion (Mac Sweeney et al., 2008) (Figure 1.4-1B). A catalytic cleft would allow BMP-1 to both confine a substrate and act upon it. Huang et al. (2009) showed that BMP-1 can bind to FN, and that this binding enhanced BMP-1 enzymatic activity. These data together lend support to
Figure 1.4-1 – BMP-1 is a multi-domain zinc- and calcium-dependent metalloproteinase. A) BMP-1 has an N-terminal protease domain, followed by 3 non-catalytic CUB (Complement/ Uegf/ BMP-1) domains, interrupted by a single EGF-like domain. The BMP-1 pre-pro-enzyme contains an N-terminal signal peptide, followed by a pro-domain that is cleaved by paired basic/ furin-like proprotein convertases in the trans-Golgi. BMP-1 is a splice variant of mTLD, which has 2 additional CUB domains and 1 additional EGF-like domain. BMP-1 has higher activity than mTLD. B) A ribbon diagram of BMP-1 with the catalytic zinc depicted as a pink sphere in the active site. BMP-1 is a V-shaped molecule with an N-terminal and a C-terminal domain divided by an active site cleft. Adapted from Sweeney et al., 2008.
our suggested mechanism of procollagen processing by BMP-1, in which FN-binding and procollagen proteolysis can occur simultaneously, while positioning procollagen adjacent to FN to promote post-processing fibrillogenesis.

Chapter 1.5 – Heparan Sulfate Proteoglycans and their ECM Interactions

Proteoglycans are considered another major component of the ECM, with almost all mammalian cells producing proteoglycans, many with the capacity to express more than one kind (Lindahl et al., 2015). Proteoglycans are comprised of a core protein with covalently attached glycosaminoglycan (GAG) chains. The GAG chains are linear, anionic polysaccharides with repeating disaccharide units that can be extensively modified with sulfation of hydroxyl groups (Ricard-Blum, 2017). Their sugar composition is the determining factor for which of four classes they are characterized as, these being keratan sulfate (KS), chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS) (Bulow and Hobert, 2006). A fifth GAG class, hyaluronan, is of special interest as it does not contain any modifications and is not attached to a core protein. Despite these distinct GAG classifications, many proteoglycans are comprised of more than one type of GAG side chain.

The GAG HS, of particular importance in the study of the ECM and FN–collagen interactions, is produced by almost all cell types, and gives rise to a collection of proteoglycans termed heparan sulfate proteoglycans (HSPGs) (Capila and Linhardt, 2002). Syndecans and glypicans are cell-surface HSPGs that are transmembrane or anchored to the membrane, respectively. Secreted HSPGs, such as agrin or perlecan, and secretory vesicle HSPGs like serglycin are prominent as well (Sarrazin et al., 2011)
(Figure 1.5-1A). To produce these HSPGs, a tetrasaccharide linker to a core protein, comprised of xylose-galactose-galactose glucuronic acid (Xyl-Gal-Gal-GlcA) is attached (Bulow and Hobert, 2006) (Figure 1.5-1B). A lone N-acetyl-D-glucosamine (GlcNAc) is added, followed by the addition of repeating disaccharide units of GlcA and GlcNAc by the copolymerase Ext1/Ext2. Ext1/Ext2 activity is immensely important, as Ext1/Ext2-null mice are embryonic lethal, and heterozygotes developed exostoses (growth of new bone on top of existing bone) (Poulain and Yost, 2015).

HSPGs, and more specifically the highly-sulfated analog of HS, heparin, have been observed to act as a template to bring two proteins together (Sarrazin et al., 2011). Importantly, heparin has been shown to increase FN-collagen binding (Johansson and Hook, 1980). FN has three binding sites for heparin, with the HepII domain, comprised of III12-14, as the main binding site on FN for heparin (Barkalow and Schwarzbauer, 1991). Heparin / HS binding to FN can induce conformational changes and reveal C-terminal cryptic binding sites for other matrix molecules, such as vascular endothelial growth factor (VEGF) (Mitsi et al., 2006). These conformational changes have been shown to remain exposed even after heparin dissociation (Mitsi et al., 2008), which is important for FN matrix assembly and insolubility (Raitman et al., 2018) (Figure 1.5-2). Procollagen / collagen has been shown to have heparin binding sites as well. Although not as distinct as the HepII domain on FN, heparin showed preferential binding near the amino terminus of both procollagen and collagen (San Antonio et al., 1994b). However, others have reported a heparin binding site with high affinity at the C-terminus of collagen (Keller et al., 1986).
Figure 1.5-1 – Heparan sulfate proteoglycans (HSPGs) and the heparan sulfate (HS) glycosaminoglycan (GAG) structure. A) HSPGs can be membrane-bound, like the transmembrane syndecans and the glypicans, which are connected to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. They can also be secreted into the ECM, like agrin and perlecan. These proteoglycans differ in the number of HS GAG chains attached to their protein core, yet are similar in the fact that they do not exist separate from the HS chains. B) HS biosynthesis begins with a linker that is attached to a serine residue on the core protein. This linker is comprised of a xylose, 2 galactose monosaccharides, and a glucuronic acid, and is identical to the linker sequence in heparin and chondroitin sulfate, another GAG. After the addition of the first N-acetylglucosamine by EXTL3, the chain is extended by the transferases EXT1 and EXT2 through alternating additions of glucuronic acid and N-acetylglucosamine. Modifications of the chain can occur during extension, with sulfation at various positions being of interest. Adapted from Poulain & Yost, 2015.
**Figure 1.5-2** – Heparin promotes FN matrix assembly. Heparan sulfate-deficient CHO-677 cells were grown for 3 days prior to supplementation with exogenous FN at 25 μg/ml +/- 50 μg/mL heparin. These cells do not produce much FN, but are not defective in FN assembly. After 24 hours, samples were fixed and immunostained for FN. FN matrix was visualized by fluorescence microscopy, and fluorescence intensities were quantified. Addition of heparin increases FN matrix assembly almost two-fold compared to FN-only treated cells. Scale bar = 50μm. *Adapted from Raitman et al., 2017.*
Chapter 1.6 – Major Questions and Thesis Objectives

ECM production requires interactions between a myriad proteins, proteoglycans, and small molecules. Investigations into the assembly of and interactions between FN and type I collagen helped to develop a deep understanding of the elegant complexities of matrix protein interactions and how FN is the master regulator of the ECM. As FN was determined to have a major collagen / gelatin binding domain at its N-terminus (Engvall et al., 1978), as well as to be required for type I collagen matrix deposition (McDonald et al., 1982), the roles for FN in collagen matrix assembly escalated. Concurrently, as procollagen / collagen synthesis, secretion, and extracellular supramolecular assembly was expounded upon, and as more binding partners and roles within the ECM for FN were discovered, research into the dynamic relationship between FN and collagen waned. This thesis aims to elucidate how procollagen processing, and nascent collagen fibrillogenesis, is remarkably linked to FN matrix assembly (Figure 1.6-1).

The principle question here is: How does the FN matrix regulate procollagen processing and nascent collagen fibrillogenesis? FN is known to be a multivalent protein that is able to interact with a number of other extracellular proteins and proteoglycans. The domains on FN that are necessary for its various interactions are usually inaccessible until FN fibril assembly begins (Xu and Mosher, 2011). The exposure of these important cryptic binding sites points towards FN matrix assembly, not simply the presence of extracellular FN, as a key process for many ECM interactions. So we ask: Does FN matrix assembly have a direct effect on procollagen processing and fibrillogenesis? Using matrix assembly inhibition assays, we can approach this question both biochemically and microscopically.
Figure 1.6-1 – Thesis objectives and major questions. A FN matrix is required for the deposition of a collagen matrix. Our data implicate FN matrix assembly in the processing of procollagen into collagen. This would also have an effect on nascent collagen fibrillogenesis, as this is carried out by self-assembly of collagen molecules. This thesis asks the question: What is the regulatory role of FN matrix assembly in procollagen processing and collagen fibrillogenesis? The following chapters will elucidate how FN interactions with procollagen propeptide proteinases affect processing, and how heparin/HS acts as a scaffold for proteins involved in processing.
If we are able to observe effects on procollagen processing when FN matrix assembly is interrupted, the question then becomes: How do the enzymes necessary for procollagen processing interact with the FN matrix? Does FN act as a template to approximate proteins involved in procollagen processing? These questions are probed using a variety of biochemical techniques including solid phase binding assays and protein pull-downs, as well as immunofluorescence microscopy.

The proteins involved in processing, whether known or hypothesized, range vastly in size. Procollagen is more than twice the length of FN, and the two proteins are considerably larger than the procollagen C-propeptide proteinase BMP-1 (Erickson and Carrell, 1983; Miller and Gay, 1987; Greenspan, 2005). Interactions between these three proteins arguably must have some other ECM component that can direct interactions. The major ECM GAGs could solve this dilemma, as HS is known to bind to almost all of the proteins important for procollagen processing (Yamada et al., 1980; San Antonio et al., 1994a; Bekhouche et al., 2010). Therefore, do extracellular HSPGs behave as scaffolds to support, or stimulate, protein-protein interactions within the procollagen processing pathway? Our approach to answer this question relies on various pull-down assays, and incorporates processing assays as well.

As we investigate these questions, we expect that our experimentation will help to determine the contributors to processing procollagen. This may alter our focus to question not simply FN matrix assembly, but how the level of FN within an ECM can affect procollagen processing. We have developed an assay that incorporates both solid-phase binding assay and suspension-based assay components to answer the question of: How does the extent of FN matrix assembly regulate how procollagen is processed? As
this thesis illuminates a new regulatory role for FN matrix assembly in procollagen protease binding and collagen processing, we hope to promote a deeper understanding of the basis of fibrotic diseases, and the development of therapeutics that target the ECM and ECM assembly in the future.
CHAPTER 2

Material and Methods
Cell culture

Cells were grown in minimum essential medium (MEM) containing 1% non-essential amino acids, 1 mM sodium pyruvate (all from Life Technologies, Grand Island, NY), and antibiotic / antimycotic cocktail (Corning Life Sciences, Oneonta, NY) supplemented with 15% fetal bovine serum (HyClone, Logan, UT) for primary human dermal fibroblasts (GM03349; Coriell Institute, Camden, NJ) or 10% fetal bovine serum for primary human lung fibroblasts (WI-38; ATCC, Manassas, VA). Cells were tested and found to be free of mycoplasma contamination. Conditioned media were collected from confluent GM03349 cells, centrifuged to remove any cells and debris, and then stored at -80°C.

Reagents

Fibronectin was purified from fresh frozen human plasma by gelatin-Sepharose affinity chromatography (Wilson and Schwarzbauer, 1992). HV120 and III9-10 GST fusion proteins were expressed and purified as described previously (Williams and Schwarzbauer, 2009). Heparin from porcine intestinal mucosa (Grade I-A, ≥180 USP units/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). Different sized heparin GAG chains, HO16 (average MW ~4650Da) and HO30 (average MW >9000Da), were obtained from Iduron (Galen Laboratory Supplies, Middletown, CT). Ascorbic acid was obtained from Sigma (L-ascorbic acid, A4403; St. Louis, MO) and used at 50 μg/mL. 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 days (Maurer et al., 2010; Hunt et al., 2012). FUD and III-11C were purified as described previously (Tomasini-Johansson et al., 2001).

Antibodies

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<th>Format</th>
<th>Clonality</th>
<th>Immunogen</th>
<th>Origin</th>
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<td>FN III1-6 domain</td>
<td>raised in-house</td>
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<td>monoclonal</td>
<td>FN III9-10 repeats</td>
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<tr>
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<td>C-terminal telopeptide of mouse type I collagen</td>
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<tr>
<td>SP1.D8</td>
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<td>amino acids 225-617 of human BMP-1</td>
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<td>6x His synthetic peptide</td>
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<tr>
<td>αGST</td>
<td>GST tag antibody</td>
<td>monoclonal</td>
<td></td>
<td>Pocono Rabbit Farm and Laboratory, Canadensis, PA</td>
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</table>

Alexa fluor-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were from Invitrogen (Eugene, OR).
**Immunoblotting**

Cells were lysed in buffered deoxycholate (DOC; 2% DOC, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA) followed by separation into DOC-soluble and DOC-insoluble fractions by centrifugation (Sechler *et al.*, 1996). Insoluble material was solubilized in 2% SDS buffer (2% SDS, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA). In some experiments, whole cell lysates were prepared by lysis in 4% SDS lysis buffer (4% SDS, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF)). Proteins were separated by SDS-PAGE on 5% polyacrylamide gels for collagen and procollagen, or 8% polyacrylamide gels for mini-procollagen, FN, BMP-1, and GST-HV120/III9-10, 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 described (Sechler *et al.*, 1996) with the following antibodies: R184 (1:50,000), 3E9 (1:8000), PA2140-2 (0.15 μg/mL), SP1.D8 (0.2 μg/mL), and αGST (1:5000). Secondary antibody was either horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:50,000 (Pierce Chemical Co., Rockford, IL). Blots were developed using Super-Signal West Pico ECL reagents (Pierce Chemical Co.). Densitometry was performed on scanned films using FIJI (Schindelin *et al.*, 2012), and exposures yielding signals within the linear range were quantified.

**Modified ELISA assays**

Cells were seeded at 4.5x10⁴ cells/cm² in tissue culture-treated 96-well plates and grown for 72 hours to confluence with 50 μg/mL ascorbic acid (Sigma, St. Louis, MO).
Cells were washed with PBS and incubated with HEPES/ Brij-35 buffer (25 mM HEPES, 0.01% w/v Brij-35, pH 7.5), 0.5 μg/mL, 1 μg/mL, 5 μg/mL, or 10 μg/mL of rhBMP-1 in PBS with 0.2 mg/mL BSA for 30 minutes at 37°C. 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 (1:10). Antibodies were detected by incubation with biotinylated goat anti-mouse IgG and streptavidin-β-galactosidase. Substrate pNPG was then added to initiate colorimetric readout, and stopped with 0.5M sodium carbonate. OD at 405 nm was determined using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA).

**Pulldown assays**

To analyze binding of BMP-1 to FN, 1 mg/mL BSA-blocked gelatin-Sepharose beads (GE Healthcare Life Sciences, Marlborough, MA) were incubated with (1) GM03349 conditioned media, +/- 1 μg/mL rhBMP-1, and +/- 5 μg/mL heparin at 4°C for 1 hour with rotation, or (2) with 20 μg/mL pFN +/- 1 μg/mL rhBMP-1, and +/-5 μg/mL or 20 μg/mL heparin in PBS 4°C for 1 hour with rotation. FN plus bound components were collected by centrifugation. Proteins were eluted with electrophoresis sample buffer (2% SDS, 96 mM Tris-HCl, pH 6.8, 2 mM EDTA, 0.01% bromophenol blue, 10.0% glycerol) 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 pulldown was accomplished using glutathione-Sepharose 4B beads (GE Healthcare Life Sciences).

To analyze binding of FN, BMP-1, procollagen, collagen, and PCPE-1 proteins to heparin, BSA-blocked heparin-Agarose beads (Sigma, St. Louis, MO) were incubated
with GM03349 conditioned medium at room temperature for 1.5 hours with rotation.

Separate GM03349 conditioned media samples were supplemented with 1 µg/mL rhBMP-1 to ensure availability of BMP-1 for heparin-binding. Washes and blocking were performed with NTE buffer (150mM NaCl, 20mM Tris-HCl, pH 7.8, 2mM EDTA). Heparin-bound components were collected by centrifugation. Proteins were eluted with electrophoresis sample buffer and analyzed by SDS-PAGE and immunoblotting.

**Immunofluorescence staining and microscopy**

For immunostaining, cells were seeded on glass coverslips in 24-well plates and grown to confluence, followed by washing with PBS and fixation in 3.7% formaldehyde in PBS. Cell densities for each experiment are listed in the table below.

<table>
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<tr>
<th>Experiment</th>
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<th>Figure</th>
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<td>8.0 x 10⁴</td>
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<tr>
<td>collagen I matrix</td>
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<td>1.3-2</td>
<td>1.1 x 10⁵</td>
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<tr>
<td>FN / collagen co-stain</td>
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<td>1.3-3</td>
<td>1.1 x 10⁵</td>
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<td>FN assembly inhibition</td>
<td>GM03349</td>
<td>3.2-1A</td>
<td>8.0 x 10⁴</td>
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<td>GM03349</td>
<td>3.2-7</td>
<td>1.1 x 10⁵</td>
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<tr>
<td>BMP-1/FN co-stain</td>
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<td>3.2-6</td>
<td>1.1 x 10⁵</td>
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<tr>
<td>Cell / FN matrix titration</td>
<td>GM03349</td>
<td>4.2-6</td>
<td>listed in figure</td>
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</table>
For BMP-1 localization, cells were incubated with 1 μg/mL rhBMP1 or equivalent volume of buffer in medium without serum, containing 5 μg/mL BSA, for 30 minutes at 37 °C, 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 μg/mL, 3E9 at 1:100, anti-6xHis at 1:500, and Alexa fluor secondary antibodies at 1:500. All antibodies were diluted in 2.0% 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

Suspension assays – For procollagen I cleavage, GM03349 standard conditioned medium was supplemented with 0.5 μg/mL rhBMP-1 +/- 5 μg/mL heparin, HO16, or HO30. For rhPro-COL1A1 time courses, rhPro-COL1A1 in dilution buffer (50 mM Tris-HCl, pH7.5, 5 mM CaCl2) was mixed with rhBMP-1 in assay buffer (50 mM Tris-HCl, pH7.5, 5 mM CaCl2, 150 mM NaCl) in a 1:3 ratio, as per manufacturer’s instructions (R&D Systems, Inc., Minneapolis, MN). Final concentrations were 18.75 μg/mL rhPro-COL1A1, 3.75 μg/mL rhBMP-1, 25 mM Tris-HCl, 2.5 mM CaCl2, and 112.8 mM NaCl, and supplemented with 5 μg/mL heparin, HO16, or HO30. Titration of heparin was used to determine an appropriate concentration for these experiments (see Figure 4.2-2B). Reactions were incubated at 37°C for up to 1 hr. Aliquots were removed from each
reaction at 15 min intervals and the reaction was stopped by addition of SDS, DTT, and EDTA to final concentrations of 2% SDS, 0.1 M DTT, and 7 mM EDTA.

*Cell culture assays* Cells were seeded at various densities (1.0x10⁴, 3.0x10⁴, 6.1x10⁴, and 12.0x10⁴ cells/ cm²) in a 96-well plate and grown for 72 hours. Cells were washed with PBS and incubated with +/- 1.0 μg/mL rhBMP-1 +/- 5 μg/mL heparin in PBS for 30 minutes at 37°C. Unbound BMP-1 and heparin was removed and 0.25 μg/mL rhPro-COL1A1 in buffer containing 50 mM Tris-HCl, pH7.5, 5 mM CaCl₂ was added and incubated for 3 hours at 37°C. rhPro-COL1A1 solution was removed, and stopped as described above.

**Immunoprecipitation**

To test production of the BMP-1 protein, GM03349 cells were plated at confluence and grown for 24 hours. Cells were washed with PBS and subject to whole cell lysis using RIPA lysis buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% DOC, 0.1% SDS, 150mM NaCl, 2mM EDTA, 50mM NaF). Cells were scraped from the plate using a rubber policeman and passed several times through a 1mL syringe attached to a 26-gauge, 3/8 inch needle prior to centrifugation at 4°C for 15 minutes at 14,000 rpm. The supernatant was collected and subjected to immunoprecipitation (IP) with 0.05M Tris-HCl, pH ≥8.0, 2.5 mM EDTA, 2.5mg/ml BSA, 2.7 mM PMSF, 0.5% DOC, 0.5% NP-40, 0.1% SDS, 5.5 μg/ml BMP-1 3E9 antibody, and Protein G beads (IP04; Millipore, Burlington, MA). IPs were subjected to centrifugation and washed with 50 mM Tris pH 8.0, 2 mM EDTA, 0.5% DOC, 0.5% NP-40, and 0.1% SDS. Captured protein was eluted
from beads with electrophoresis sample buffer and separated by SDS-PAGE prior to immunoblotting using BMP-1 3E9 antibody. Secretion of BMP-1 was tested through IP of GM03349 conditioned media collected from plates prior to RIPA lysis. IPs were executed and immunoblotted as previously described.

Statistical Analysis

Densitometry results are reported as the mean +/- standard error for a minimum of 3 samples, unless specified otherwise. Values for analysis were collected using FIJI software. Means were computed using Microsoft Excel (v.16.24) and compared using a two-tailed Student’s t-test, with p < 0.05 considered statistically significant.
CHAPTER 3

Characterization of the Interaction between BMP-1 and Fibronectin

Materials from this chapter have been presented at:
The American Society for Matrix Biology 2018 Biennial Meeting in Las Vegas, NV.

Some of the work presented in this chapter has been submitted for publication in:
Chapter 3.1 – Introduction

The assembly of a FN matrix is an essential biological process. It provides a foundation upon which the remainder of the ECM can be fabricated. In the interstitial ECM, type I collagen and FN are frequently found together. Type I collagen can bind to FN, and a previously established FN matrix is required for collagen matrix deposition (Velling et al., 2002). This relationship is not unique to type I collagen. ECM deposition of collagens 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; Sottile et al., 2007; Singh et al., 2010; Miller et al., 2014). Since 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. With this being the case, it is not surprising to propose that the FN matrix may play a role in the processing mechanisms that generate a functional, fully processed collagen molecule.

Type I collagen is expressed as the proprotein procollagen. This precursor molecule has propeptides at both termini that require cleavage by specific extracellular proteinases. The globular N-terminal propeptide is cleaved by ADAMTS-2, while BMP-1 processes the pseudo-cruciform C-terminal propeptide (Greenspan, 2005). 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; Prockop and Hulmes, 1994). Therefore N-propeptide cleavage, and thus ADAMTS-2 activity, is not required to initiate fibrillogenesis. Since C-propeptide cleavage is crucial to both the processing and fibrillogenesis of procollagen, we have chosen to concentrate on BMP-1 and its role in FN matrix-based procollagen processing.

BMP-1 is a known binding partner of FN. Work by Huang et al. (2009) showed that BMP-1 can bind to both cellular and plasma FN. Additionally, they showed that BMP-1 binding to FN increases the proteinase’s activity. BMP-1 and FN also share heparin binding activity. Heparin is a highly sulfated glycosaminoglycan that is highly related to heparan sulfate (HS). It plays a role in diverse biological processes such as anticoagulation, where it inhibits thrombin and prevents blood clotting (Sarrazin et al., 2011). Heparin binds FN at the HepII domain, which covers FN type III repeats 12 through 14 (III\textsubscript{12-14}) (see Figure 1.2-1) (Barkalow and Schwarzbauer, 1991). Heparin also binds to type I collagen and has been observed to enhance the rate of binding of FN to immobilized collagen (Johansson and Hook, 1980). As heparin/HS is a known binding partner of all three molecules - FN, BMP-1, and collagen - it is possible that heparin plays a role in regulating or promoting their interactions.

In this chapter, I describe biochemical and microscopic approaches used to analyze the localization and binding of BMP-1 to FN and to a FN matrix. Additionally, recombinant FN fragments allowed me to map a binding site for BMP-1 on FN and to test the effects of heparin on binding. My results show that FN, BMP-1 and heparin form
a ternary complex and I propose a model to explain the relationship between this complex and interactions with type I procollagen.

Chapter 3.2 – Results

Dependence of type I procollagen 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 a bacterial peptide, FUD, that binds to the assembly domain of FN and inhibits the formation of FN fibrils (Maurer et al., 2010) also eliminates collagen I assembly (Figure 3.2-1A). These results confirm that, as with other cell types (Velling et al., 2002; Sottile et al., 2007), these fibroblasts require a FN matrix for collagen I deposition.

During collagen fibril formation, N- and C-propeptides are cleaved by extracellular proteases. 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 -insoluble fractions (Figure 3.2-1B). Collagen cleavage products lacking one (pN/Ccoll) or both (coll) propeptides are evident in the DOC-insoluble fractions. In contrast, inhibition of FN assembly in FUD-treated cells results in a significant reduction of collagen in the DOC-insoluble sample. Whole cell lysates prepared with an SDS lysis buffer demonstrate the lack of procollagen proteolytic processing when FN assembly is inhibited.
**Figure 3.2-1** – 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 co-stained 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 = 10μm. 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% PAG and immunoblotted with anti-COL1A1 (top) or anti-FN (R184). Locations of procollagen, pN- or pC-collagen, and cleaved collagen I are indicated (right) and molecular mass standards are indicated on the left.
(Figure 3.2-1B). 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 a FN matrix suggests that FN matrix may have a role in localizing procollagen and/or procollagen proteases to promote proteolytic cleavage and collagen fibrillogenesis. Since cleavage of the C-propeptide is required for collagen fibrillogenesis, we chose to focus on interactions with the procollagen C-propeptide proteinase BMP-1.

BMP-1 binding to soluble FN

To probe interactions between BMP-1 and FN, we performed a gelatin-Sepharose pulldown to isolate FN from GM03349 conditioned media supplemented with 1 μg/mL of a recombinant human BMP-1 (rhBMP-1). FN that is captured by the gelatin on the beads allows analysis of not just FN in a sample, but of FN binding partners as well. Immunoblots of eluates from the pulldown were probed for BMP-1 and confirmed that rhBMP-1 binds to endogenous soluble FN in the media (Figure 3.2-2A). Due to the presence of other proteins, growth factors, proteoglycans, etc., in conditioned media, these results do not determine if the interactions are direct or indirect. To probe direct interactions between BMP-1 and FN, we performed a gelatin-Sepharose pulldown using 1.0 μg/mL rhBMP-1 and 20 μg/mL human plasma FN (hpFN) in buffered solution. An immunoblot of eluates from the pulldown was probed for BMP-1 and shows that rhBMP-1 directly binds to FN in solution but does not bind to gelatin-Sepharose in the absence of added FN (Figure 3.2-2B).
Figure 3.2-2 – BMP-1 binds soluble FN. A) FN was pulled down from GM03349 conditioned media after 1 hr incubation with (+) or without (-) the addition of 1.0 μg/ml rhBMP-1 using gelatin-Sepharose beads. Bound BMP-1 was detected by immunoblotting with anti-BMP-1 antibody. 10 ng of pure rhBMP-1 was electrophoresed in the right-hand lane. B) 20 μg/ml FN in PBS was mixed with 1.0 μg/ml rhBMP-1 +/- 5.0 μg/ml heparin and incubated for 1 hour. FN was pulled down and eluates were analyzed as in (A). 2 ng of pure rhBMP-1 was electrophoresed in the right-hand lane. Molecular mass markers are indicated at the left.
Effects of heparin on BMP-1 interactions with the FN matrix

Heparin/HS is a known binding partner for both FN and BMP-1 (Hynes, 1990; Bekhouche et al., 2010). This led us to question if heparin has a role in BMP-1-FN interactions. To ensure that heparin can in fact bind FN and BMP-1, we performed a heparin-agarose pulldown on GM03349 conditioned media supplemented with 1.0 μg/mL rhBMP-1, as GM03349 cells do not produce sufficient quantities of the proteinase. An immunoblot of the eluate from the pulldown was probed for either FN or BMP-1 and shows that the heparin beads captured both proteins (Figure 3.2-3A). We then performed a gelatin-Sepharose pulldown of endogenous FN in GM03349 conditioned media supplemented with 1.0 μg/mL rhBMP-1 in the presence or absence of 5.0 μg/mL heparin. The immunoblot in Figure 3.2-3B shows a slightly higher level of BMP-1 in the eluate with the addition of heparin compared to no heparin.

Because there are many different heparin binding proteins contributed by the serum in the conditioned medium, the effects of heparin on FN-BMP-1 binding in the gelatin pulldown was repeated with purified proteins. Gelatin-Sepharose was used to capture 20 μg/mL purified hpFN supplemented with 1 μg/mL rhBMP-1, and 5 μg/mL heparin in a buffered solution. The immunoblot shows significantly more BMP-1 in the eluate with both FN and heparin than with FN alone (Figure 3.2-3C). These results indicate that the interactions between FN, BMP-1, and heparin are direct. Furthermore, they show that BMP-1 binding to FN is stimulated by heparin.

BMP-1 reportedly has multiple binding sites on FN (Huang et al., 2009), so GST-tagged recombinant FN fragments were used to identify sites of interaction. The fusion protein GST-HV120 contains the entire HepII domain inclusive of the variable region
Figure 3.2-3 – Heparin promotes rhBMP-1 binding to FN. A) Heparin-agarose beads were mixed with GM03349 conditioned media supplemented with 1.0μg/mL rhBMP-1 and incubated for 1.5 hours. Samples were eluted using electrophoresis sample buffer and immunoblotted with an anti-FN or anti-BMP-1 antibody. B) GM03349 conditioned media containing cell-produced FN with or without the addition of 1.0 μg/ml rhBMP-1 and 5.0μg/ml heparin (as indicated above the blot) were incubated for 1 hour. FN was pulled down using gelatin-Sepharose beads and bound BMP-1 was detected by immunoblotting with anti-BMP-1 antibody. C) 20 μg/ml FN in PBS was mixed with 1.0 μg/ml rhBMP-1 (10:1 molar ratio) +/- 5.0μg/ml heparin and incubated for 1 hour. FN was pulled down and analyzed as in (B). Data are representative of 3 experiments. Molecular mass markers are indicated.
and III15. GST-III9-10 contains two type III modules from the integrin binding domain (see Figure 1.2-1). We found that BMP-1 binds to GST-HV120 but not to GST-III9-10 in a glutathione-Sepharose pulldown experiment (Figure 3.2-4A). As with the FN pulldowns, addition of heparin to the GST-fragment binding assays shows that heparin promotes rhBMP-1 binding to the HepII domain of FN but has no effect on binding to III9-10 (Figure 3.2-4A). The efficiency of the pulldowns was confirmed by immunoblotting for the recombinant fragments using anti-GST antibodies which shows equivalent amounts of GST proteins in the eluates (Figure 3.2-4B). These results map a BMP-1 binding site to FN’s HepII domain and demonstrate enhancement of this binding by heparin.

**BMP-1 binding to the FN matrix**

BMP-1 interacts with FN and the HepII domain of FN in solution but this proteinase would normally function in the context of an ECM. To measure its interaction with a FN-rich ECM, a modified enzyme-linked immunosorbent assay (ELISA) was performed after incubation of 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 and 10 μg/ml (Figure 3.2-5). A parallel ELISA with anti-FN antibodies demonstrates the presence of a FN matrix in these cultures.

To examine the distribution of the proteinase in the matrix, fibroblasts were grown to confluence prior to incubation with 1.0μg/mL rhBMP-1. Cells were then immunostained for BMP-1 and FN. Fluorescence confocal images of cultures without added BMP-1 show FN fibrils interspersed with anti-BMP-1 staining that does not co-localize with FN staining. In the presence of BMP-1, however, there are many sites of co-
Figure 3.2-4 – Heparin promotes BMP-1 binding to the HepII domain of FN. A) 1.0 μg/mL of recombinant GST-tagged HepII domain of FN was mixed with 1.0 μg/ml rhBMP-1 +/- 5.0 μg/ml heparin and incubated for 1 hour. GST-III9-10 was used as a control. GST proteins were pulled down with glutathione beads and immunoblotted with anti-BMP-1 antibodies. 2.0 ng of pure rhBMP-1 was electrophoresed in the right hand lane. B) Equal aliquots of eluates brought down with glutathione beads (from panel A) were immunoblotted with anti-GST antibodies. Equivalent amounts of eluted proteins show the efficiency of the pulldown assay.
Figure 3.2-5 – BMP-1 binds to the FN matrix. 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 3 experiments +/- S.E.M. * p < 0.05 compared to lowest BMP-1 concentration.
Localization of the anti-BMP-1 staining with FN fibrils (Figure 3.2-6 top). The rhBMP-1 contains a His tag so immunostaining was repeated with an anti-His antibody. Background staining in the absence of added BMP-1 was reduced compared to the anti-BMP-1 antibody background (compare Figure 3.2-6 left, top and bottom). Importantly, anti-His staining of rhBMP-1 also shows co-localization with FN fibrils (Figure 3.2-6, bottom right). These data indicate that BMP-1 co-localizes with FN fibrils, and also support the suggestion that BMP-1 associations with the ECM are at least partly through its binding to FN.

Localization of type I procollagen and type I collagen in the matrix

The localization of BMP-1 to FN fibrils, along with the co-localization of collagen and FN in a matrix (Velling et al., 2002), and our data on procollagen processing in the absence of a FN matrix (Figure 3.2-1B) led us to question if procollagen localizes with FN matrix in its early stages of development. 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; Prockop and Hulmes, 1994). GM03349 fibroblasts were grown for 24 hours to provide sufficient time for them to assemble nascent collagen fibrils. Co-immunostaining with antibodies against the N-propeptide of type I procollagen and FN show a high degree of co-localization between fibrils of pNcollagen and FN (Figure 3.2-7A). This co-localization suggests that FN and procollagen are adjacent to each other at a time when collagen processing is occurring.
Figure 3.2-6 – BMP-1 co-localizes with FN fibrils. GM03349 cells at confluence were incubated with medium alone (left) or medium with 1.0 μg/mL rhBMP-1 (right) and then fixed and co-stained 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. White arrows indicate some regions of co-localization. Scale bar = 5 μm.
Figure 3.2-7 – Procollagen co-localizes with FN fibrils. GM03349 cell cultures at confluence were co-immunostained for FN and either (A) pN-collagen (pro) 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 = 10μm
Immunostaining with an antibody that detects total type I collagen (processed and unprocessed) shows a lower degree of co-localization with FN fibrils compared to procollagen staining (Figure 3.2-7B). 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.

Since both procollagen and BMP-1 localize to FN fibrils, and BMP-1 binding to FN enhances proteolytic activity (Huang et al., 2009), along with heparin promoting BMP-1 binding to FN, we propose a role for FN matrix in procollagen processing where heparin/HS conveys BMP-1 to its binding site on FN, whereupon it can process procollagen.

**Chapter 3.3 – Discussion**

In this chapter, we determined how FN matrix and its assembly influence the localization of BMP-1 by providing sites where the proteinase can bind for processing procollagen. To do so, we utilized a primary human dermal fibroblast cell line (GM03349) that assembles a robust matrix rich in FN and type I collagen, but does not highly express BMP-1. This enabled us to regulate the concentration of the proteinase through supplementation with a purified recombinant human BMP-1 (rhBMP-1). We show that both type I procollagen and rhBMP-1 co-localize with FN matrix fibrils and inhibition of FN matrix assembly eliminates proteolytic processing of procollagen. As BMP-1 is a known binding partner of FN, and FN binding enhances BMP-1 activity (Huang et al., 2009), we propose that the FN matrix acts as a scaffold that positions proteinase and substrate together to promote cleavage. Additionally, BMP-1 has been
shown to bind to FN at multiple sites 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.

Since heparin/HS is a known binding partner of FN, and BMP-1, we investigated if heparin-FN associations play a role in FN-BMP-1 interactions. We found that the presence of heparin promoted BMP-1 binding to FN, and we mapped a BMP-1 binding site to within the HepII domain of FN (repeats III12-14), which is the main heparin binding site of FN (Barkalow and Schwarzbauer, 1991). This would make it possible for heparin to act as a bridge to link BMP-1 and FN. In the case of a 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 since 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).

Interestingly, the variable/IIICS region of FN, which is included in the recombinant FN fragment GST-HV120 used to map the BMP-1 binding site, has been shown to be a zinc-binding module, capable of binding a single zinc ion (Askari et al., 2007). BMP-1 is a zinc metalloendopeptidase, requiring a Zn²⁺ ion in its active site for proteolytic activity (Gomis-Ruth, 2003). Zinc concentrations that exert functional effects are submillimolar, and with reported values in serum ranging from 15µM to 100µM and reaching millimolar levels in the liver, zinc’s influence on FN-protein interactions is physiologically relevant (Iyengar and Woittiez, 1988). Thus, the variable region might
Figure 3.2-8 – 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., 1994b).
behave as a Zn$^{2+}$ ion sink for BMP-1, where the proteinase can then bind to FN and be activated for proteolytic cleavage of procollagen.

Another possibility is that heparin 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; Mitsi et al., 2008; Li et al., 2015; Vogel et al., 2016; Raitman et al., 2018). 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.

Heparin might also have a procollagen-specific role by stabilizing its interactions with FN. 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 3.2-8). 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.
CHAPTER 4

Procollagen Processing is Dependent on BMP-1 Binding to Fibronectin Matrix

Materials from this chapter have been presented at:
The American Society for Matrix Biology 2018 Biennial Meeting in Las Vegas, NV.

Some of the work presented in this chapter has been submitted for publication in:
Chapter 4.1 – Introduction

Type I procollagen processing in the extracellular space relies on the activity of specific metalloendopeptidases that cleave the propeptides at either end of the molecule (Greenspan, 2005). The C-terminal propeptide is processed by the proteolytic activity of BMP-1. The activity of this C-propeptide proteinase is essential for procollagen maturation that leads to fibrillogenesis (Canty and Kadler, 2005). In vitro studies have shown that cleavage of the C-propeptide by BMP-1 is enough to initiate pNcollagen self-assembly (Prockop and Hulmes, 1994). Thus, the activity of the N-propeptide proteinase ADAMTS-2 is not required for fibrillogenesis. Electron microscopic studies into procollagen processing and assembly in tendons have shown that BMP-1 activity occurs in plasma membrane invaginations where ordered procollagen aggregates are processed together before being released into the ECM (Canty et al., 2004). This localization of procollagen processing has only been demonstrated in tendon. However, proBMP-1 cleavage by a furin-like convertase can occur in the Golgi apparatus raising the possibility that BMP-1 might act on substrates prior to secretion (Leighton and Kadler, 2003). It appears as though a comprehensive understanding of the regulation of BMP-1 is needed.

Our analysis of FN matrix interactions with the C-propeptide proteinase BMP-1 shows localization to and association with FN through its Hep II domain, and that heparin/HS plays a role in promoting these interactions. As we have shown that procollagen colocalizes with FN fibrils as well, we propose C-propeptide cleavage by BMP-1 is FN fibril-associated and heparin/HS can augment this process. Heparin has already been shown to play a role in collagen fibrillogenesis by inhibiting the oxidation
of lysine residues necessary for intermolecular cross-linking (Gavriel and Kagan, 1988). Others have shown a role for HSPGs in localizing propeptide proteinases to their collagenous substrate to promote processing (Bekhouche et al., 2010). Additionally, heparin/HS can act as a scaffold to promote protein interactions (Sarrazin et al., 2011), and it has been reported to promote FN-collagen interactions (Johansson and Hook, 1980). To investigate the role of FN and heparin/HS in the cleavage of the procollagen C-propeptide by BMP-1, we developed and employed a procollagen processing assay that utilizes endogenous or recombinant procollagens for cleavage reactions in solution and in association with a cell-assembled ECM.

Chapter 4.2 – Results

Determining BMP-1 expression in dermal fibroblasts

BMP-1 localization and binding was examined in the previous chapter through supplementation with a recombinant BMP-1 (rhBMP-1), which led us to question if the GM03349 cells produce BMP-1 in sufficient quantities to allow for observing procollagen processing. In order to answer this question, we performed a DOC solubility assay on GM03349 cell cultures to separate soluble nascent FN and collagen fibrils from insoluble stable fibrils. DOC lysates were immunoblotted with an anti-type I collagen antibody that detects all forms of collagen (Figure 4.2-1, bottom panel). The DOC-insoluble fraction contained fully processed collagen, but this form was not detected in soluble fractions. The DOC-soluble fraction and conditioned medium contained only procollagen with both propeptides and an intermediate band that is the appropriate size.
Figure 4.2-1 – Procollagen C-propeptide processing by BMP-1. GM03349 cells were grown to confluence and solubilized with a deoxycholate (DOC) detergent-based buffer. Insoluble proteins were separated through centrifugation, and subsequently solubilized in a SDS buffer. DOC soluble (sol), DOC insoluble (insol) and conditioned media samples were immunoblotted with either anti-procollagen N-propeptide antibody (Npp-COL1A1, top panel) or anti-type I collagen antibody (COL1A1, bottom panel). Locations of procollagen (pro), pNcollagen (pNcol), and pCcollagen (pCcol) are indicated to the right. Molecular mass markers are on the left.
for collagens lacking one of the propeptides. pNcollagen and pCcollagen have similar molecular weights.

Detection of collagens using an anti-procollagen N-propeptide antibody that recognizes the globular N-terminal propeptide of type I procollagen was used to assess levels and distribution of procollagen intermediates. Unprocessed and partially processed procollagens containing the N-propeptide were detected in all three cell fractions (Figure 4.2-1, top panel). The intensity of the procollagen intermediate-sized band is drastically diminished in intensity across all samples compared to the total collagen immunoblot. This comparison reveals that a majority of the procollagen intermediates are pCcollagen, in which the N-propeptide has been cleaved by ADAMTS-2. This result suggests that BMP-1 expression is low. In support of this idea, we were unable to definitively identify BMP-1 by either immunoblots or immunoprecipitations of GM03349 cell or media fractions (not shown). It is reported that human dermal fibroblasts have low expression of BMP-1 (Uhlen et al., 2015). We conclude that rhBMP-1 supplementation is required in the processing assays, as with our localization and binding assays.

**Determination of rhBMP-1 and heparin concentrations for application in processing assays**

In order to determine the amount of supplemental rhBMP-1 to use in our assays, we performed a titration with increasing concentrations of rhBMP-1 added to GM03349 conditioned media containing endogenous procollagen and analyzed cleavage after incubation for 15 minutes. The immunoblot using an anti-procollagen N-propeptide antibody shows that partial processing of procollagen is first observed at 0.5μg/mL with
Figure 4.2-2 – Titrations of BMP-1 and heparin in the procollagen processing assay. GM03349 conditioned medium was mixed with increasing concentrations of rhBMP-1 (top) or 0.5 μg/mL rhBMP-1 plus increasing amounts of heparin (bottom) as indicated. Samples were incubated for 15 minutes and reactions stopped through the addition of a stop solution of SDS, DTT, and EDTA. Samples were immunoblotted with an anti-procollagen N-propeptide antibody. Locations of procollagen and pN-collagen are indicated (right) and molecular mass markers are on the left.
increasing cleavage at higher concentrations (Figure 4.2-2 top). These results also show that rhBMP-1 is active in this assay. 0.5μg/mL BMP-1 was selected for use in our processing assays.

We have shown that heparin promotes binding of BMP-1 to FN, and we hypothesize that it may also promote procollagen processing by rhBMP-1. To determine a concentration with which to supplement processing assays, we performed a titration with heparin in the presence of rhBMP-1. Increasing concentrations of heparin were added to GM03349 conditioned media supplemented with 0.5μg/mL rhBMP-1 and incubated for 15 minutes as per the rhBMP-1 titration. The results show that procollagen processing is stimulated at even the lowest concentration of heparin added (Figure 4.2-2 bottom). As the concentration of heparin reaches 3.0μg/mL, there is little observable unprocessed procollagen and only the pNcollagen intermediate remains. 5 μg/mL was selected to use in procollagen processing assays, as it provided the maximal effect.

**Proteolytic cleavage of type I procollagen by BMP-1 in solution**

To test whether heparin binding to BMP-1 affects processing of endogenous procollagen, we treated GM03349 cell conditioned medium containing cell-produced type I procollagen and supplemented with rhBMP-1 alone at 0.5μg/mL or with rhBMP-1 plus heparin at 5 μg/mL. Proteolytic processing of endogenous procollagen was monitored over time by immunoblotting with an anti-procollagen I N-propeptide antibody. Control samples of conditioned medium with or without heparin showed no observable procollagen processing by the conclusion of the time course (Figures 4.2-3A, 3C). Treatment with rhBMP-1 alone shows a time-dependent generation of the
Figure 4.2.3 – Heparin promotes processing of cell-produced procollagen by rhBMP-1. A-D) GM03349 conditioned medium was mixed with +/- 0.5μg/mL rhBMP-1 +/- 5.0μg/mL heparin. Aliquots were removed from each reaction at the indicated time and stopped with a solution of SDS, DTT, and EDTA. Samples were immunoblotted with anti-procollagen N-propeptide antibody. Untreated GM03349 conditioned media were electrophoresed in the first lanes. Locations of procollagen (pro) and pNcollagen (pNc) are indicated to the right of each blot. Molecular mass markers are to the left of each panel. E) Densitometry results for panels B & D. Values acquired were normalized to rhBMP-1 – heparin “t0 procollagen” for each experiment. Normalized values from three experiments were averaged to give fold change ± SEM. * p < 0.05 compared to rhBMP-1 – heparin “t0 procollagen”.

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<td>pNcollagen</td>
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*p < 0.05
pNcollagen band and reduction in the type I procollagen band (Figure 4.2-3B). After 15 minutes, about half of the procollagen was intact, and at the conclusion of the time course, detectable unprocessed procollagen remained (Figure 4.2-3E). Supplementation with heparin significantly enhanced cleavage with no detectable procollagen after only 15 minutes (Figure 4.2-3D). Quantification confirms that heparin stimulates procollagen processing by rhBMP-1 compared to rhBMP-1 alone at 15 min (Figure 4.2-3E), thus demonstrating that heparin increases the rate of procollagen cleavage by BMP-1.

To examine 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 minutes with a slight band of unprocessed rhPro-COL1A1 remaining at the conclusion of the experiment (Figure 4.2-4B). In the presence of heparin, cleavage by BMP-1 was completed in less than 30 minutes (Figure 4.2-4D). Control samples of rhPro-COL1A1 with or without heparin showed no observable procollagen processing by the completion of the time course (Figures 4.2-4A, 4C). 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.

Contribution of heparin chain length to processing of procollagen by rhBMP-1

Heparin/HS chains can vary in length and longer chains have the potential to interact with multiple molecules. For example, the minimum binding size for FN’s HepII
Figure 4.2-4 – Heparin interacts with rhBMP-1 directly to enhance procollagen cleavage in solution. A-D) rhPro-COL1A1 was mixed with +/- 0.5μg/mL rhBMP-1 +/- 5.0μg/mL 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 were immunoblotted with anti-procollagen N-propeptide antibodies. Locations of mini-procollagen (mini-pro) and mini-pN-collagen (mini-pNc) are indicated to the right of each blot. Molecular mass markers are to the left of each panel. Blots are representation of 4 experiments.
domain is 16 saccharides (Ingham et al., 1990; Walker and Gallagher, 1996). The unfractionated heparin used in these experiments is 72 saccharides long, on average, and can therefore bind 4-5 HepII domains simultaneously. We questioned if the enhancing ability of the unfractionated heparin could be attributed to chain length and multimeric interactions. We utilized two fractionated heparins of distinct chain length, HO16 (16 sugars) and HO30 (30 sugars) in our conditioned media processing reactions. In a single incubation period of 15 minutes, proteolytic processing of endogenous procollagen was complete with the addition of HO30 but partial with HO16 addition (Figure 4.2-5A). The addition of either HO16 or HO30 alone had no effect on processing and appear like the control medium. rhBMP-1 + HO16 gave partial processing of procollagen, appearing almost identical to rhBMP-1 alone. These data indicate that heparin chain length does influence processing of procollagen. We then performed a processing time course with HO16 and HO30. Control samples of conditioned medium with or without HO16 / HO30 showed no observable procollagen processing by the conclusion of the time course (Figures 4.2-5B, leftmost panels). Treatment with rhBMP-1 + HO16 shows a time-dependent generation of the pNcollagen band and reduction in the type I procollagen band (Figure 4.2-5B, top right panel). However, after 45 minutes, detectable unprocessed procollagen remained. rhBMP-1 + HO30 presented with significantly enhanced cleavage compared to HO16 with no detectable procollagen after only 15 minutes (Figure 4.2-5B, bottom right panel). These data show that heparin chain length has an impact on the rate of procollagen cleavage and that HO30, but not HO16, heparin stimulates the processing of procollagen similarly to unfractionated heparin. This finding implicates the increased
**Figure 4.2-5** – Heparin chain length affects procollagen processing. GM03349 conditioned medium was mixed with +/- 0.5μg/mL rhBMP-1 + 5.0μg/mL HO16 or HO30 and incubated for (A) 15 minutes or (B) times as indicated. After the incubation, each reaction was stopped with a solution of SDS, DTT, and EDTA and immunoblotted with anti-procollagen N-propeptide antibodies. Untreated GM03349 conditioned medium was electrophoresed in the first lanes. Locations of procollagen (pro) and pN-collagen (pNcol) are indicated to the right of each blot. Molecular mass markers are to the left of each panel. Blots are representative of 3 experiments (A) and 2 experiments (B).
number of binding sites on longer heparin chains in determining the rate of procollagen processing by BMP-1.

**Procollagen processing by matrix-bound BMP-1**

Our results in the previous chapter show co-localization of procollagen and BMP-1 with FN fibrils. To determine if this localization supports procollagen processing, we tested the ability of rhBMP-1 bound to a FN matrix to cleave rhPro-COL1A1. GM03349 fibroblasts were seeded at increasing densities to yield cultures with increasing amounts of FN matrix (Figure 4.2-6). 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 hours. Aliquots were immunoblotted using an anti-procollagen I N-propeptide antibody. No cleavage was detected in the untreated control and with the addition of heparin only (Figure 4.2-7A, 7C). rhBMP-1-only treated samples show very little cleavage at the lowest cell densities but show increasing rhPro-COL1A1 cleavage at higher FN matrix levels (Figure 4.2-7B). Quantification confirms that cleavage primarily occurs at the two highest cell densities (Figure 4.2-7E). 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 at even the lowest cell density (Figure 4.2-7D). Taken together, these results show that, as with BMP-1 in solution, heparin also enhances type I procollagen cleavage by matrix-bound BMP-1.
Figure 4.2-6 – GM03349 cell densities and matrix levels. (Top, BF) Cells were plated at the indicated cell densities and phase images were captured after 72 hr of growth. Cells are confluent at the highest densities. (Bottom, FN) Parallel samples were fixed and stained with R184 anti-FN antiserum followed by fluorescent goat anti-rabbit secondary. The amount of FN matrix increased with cell density.
Figure 4.2-7- Heparin enhances processing of procollagen by matrix-bound BMP-1. GM03349 cells plated at various densities (cells/cm²) were grown for 72 hours to yield increasing amounts of FN matrix indicated by the black triangle (see Supp. Fig. S5). Cultures were then incubated with rhBMP-1 (A) or rhBMP-1 + heparin (B) followed by rhPro-COL1A1 for 3 hr. The rhPro-COL1A1 solution was collected and equal aliquots were immunoblotted with an anti-procollagen N-propeptide antibody to observe processing of rhPro-COL1A1. 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 4 experiments. 48 hour processing experiments yielded similar results. E) Densitometry results for panels B & D. Values acquired were normalized to the processed band at the highest cell density (12.0 x 10⁴ cells/cm²) within each condition. Data from four experiments were averaged to give fold change ± SEM. *p < 0.05 compared to value at 12.0 x 10⁴ cells/cm² within each condition. Similar results were obtained with cells grown for 48 hr (not shown).
Chapter 4.3 – Discussion

In this chapter, we analyzed the role of FN matrix in procollagen cleavage by BMP-1, its C-propeptide proteinase, utilizing human dermal fibroblasts that assemble a robust ECM. We show that heparin enhances BMP-1 proteolytic activity in solution, and, using purified components, that this enhancement is a result of direct interactions between procollagen, BMP-1, and heparin. Additionally, we show that heparin chain length contributes to processing. Further we showed that in an ECM microenvironment, cleavage of type I procollagen by BMP-1 scales with the FN level in the matrix. Moreover, the presence of heparin promotes BMP-1-mediated processing in matrices with low FN levels. Taken together, our results suggest that (I) the FN matrix acts as a scaffold that binds and positions proteinase and substrate together to promote procollagen cleavage, and (II) that heparin enhances that process perhaps by acting as a linker to bridge FN, procollagen, and BMP-1.

Heparin/HS chains of sufficient length are known to bring two or more proteins together and promote interactions. We have previously shown that a single unfractionated heparin chain binds to multiple FN molecules, a HO30 chain binds two, and a HO16 chain binds to one FN (Raitman et al., 2018). This property of heparin is likely to explain the ability of heparin to promote procollagen processing in our solution assay. We propose that in this assay heparin acts as a scaffold, binding BMP-1 and procollagen simultaneously and thus increasing the rate of procollagen cleavage. This idea is supported by our results with short chain (HO16) heparin which did not promote cleavage and long chain (HO30) heparin which did. Based on HO30 binding to FN, we suggest that a heparin chain of this length is able to bind both BMP-1 and procollagen.
and is therefore acting similarly to unfractionated heparin to promote processing. HO16, on the other hand, seems unable to bind multiple proteins fitting with its lack of activity in our solution assay.

Our cleavage data suggest that GM03349 cells produce more N-propeptidase than BMP-1 (C-propeptidase) since the level of pCcollagen was higher than pNcollagen in DOC lysates. These cells also express the metalloproteases meprin α and meprin β, which reportedly cleave both propeptides from procollagen (Broder et al., 2013). While it is possible that these proteinases contribute to collagen processing and fibrillogenesis, they are not likely to be major contributors to the BMP-1 cleavage reported here since we controlled for endogenous activities in all of our experiments.

The processing experiments in solution proved to be informative about interactions taking place during procollagen processing. However, FN in a matrix is not soluble, and in solution there are cryptic binding sites that are not accessible. Examination of procollagen processing in an ECM microenvironment showed increased procollagen cleavage by BMP-1 as FN matrix levels increased. We propose that this is the result of more FN-bound BMP-1. With the addition of heparin, we observed a further increase in processing at even the lowest FN matrix levels. Heparin may act as a tether to bring more BMP-1 molecules to the FN matrix. Because heparin/HS also binds to collagen, it may stabilize procollagen interactions with FN in proximity to the FN-bound BMP-1. In that case, the ternary complex proposed in Chapter 3 would become a hub for procollagen cleavage within the FN matrix. In fact, these multimeric interactions may be a natural process for increasing the local concentration of proteinases and substrates,
which has been shown to be artificially accomplished through macromolecular crowding with large neutral polymers like dextran sulfate (Chen and Raghunath, 2009).
CHAPTER 5

Model for Collagen Processing and Fibrillogenesis Dependent on FN Matrix

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The American Society for Matrix Biology 2018 Biennial Meeting in Las Vegas, NV.

Some of the work presented in this chapter has been submitted for publication in:
Chapter 5.1 – Introduction

The assembly of a FN matrix provides a foundation for the entirety of the ECM to be built upon. FN’s multidomain structure allows for interactions with a multitude of extracellular proteins, proteoglycans, growth factors, and signaling molecules that can lead to, or are a part of, vital biological processes including cell differentiation and wound healing. FN is considered the master regulator of the ECM, a role best exemplified by FN-collagen interactions. Despite being the most abundant ECM protein that provides structure and support to tissues, a type I collagen matrix will not be deposited unless there is a FN matrix assembled (McDonald et al., 1982). This leads us to question in what other parts of the procollagen maturation and collagen fibrillogenesis pathway does FN play a regulatory role?

Chapter 5.2 – Proposed Models

*Processing in solution*

BMP-1 is able to cleave the C-propeptide of procollagen I in solution in the absence of other proteins. The addition of heparin increases the rate of that reaction showing that these three molecules alone are sufficient for a maximum rate of cleavage (Figure 5.2-1). Under these conditions, 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. This mechanism is supported by our binding data and the effects on processing of heparin chains that bind one, two, or more proteins at once. The shortest heparin chain (HO16) can only bind BMP-1 or procollagen but not both and had no effect on the rate of cleavage. HO30 and longer heparin chains accelerated cleavage. The most
Figure 5.2-1 – Model for FN matrix-independent procollagen processing. In solution, BMP-1 cleaves procollagen at a certain rate (red in left clock) that is dependent on BMP-1 concentration. The addition of heparin decreases the time for procollagen conversion (right clock). Solution assays either contained soluble FN, as in conditioned medium, or lacked FN as with rhPro-COL1A1 processing experiments.
likely explanation is through molecular bridging of enzyme with substrate. Another possibility is the concept of local concentration amplification via macromolecular crowding. Through the use of very long dextran sulfate chains (> 500 kDa), the excluded volume effect (EVE) was exploited to increase the local concentration of molecules involved in collagen processing and fibrillogenesis (Lareu et al., 2007). Addition of dextran sulfate to cultured fibroblasts increased the amount of cleaved collagen, most of which was then found in association with cells as opposed to in the culture medium. In addition to collagen deposition, EVE has been shown to accelerate lysyl oxidase (LOX)-mediated fibril cross-linking allowing a full collagen matrix to be assembled in as little as two days depending on the macromolecular crowder used (Chen et al., 2009). These so-called “scar-in-a-jar” studies have been used to recapitulate some of the ECM deposition events observed in pathophysiological conditions associated with scarring and fibrosis. Through these studies, BMP-1 is considered a “tardy” proteinase in vitro, meaning that under standard cell culture conditions, fibroblasts can only deposit a limited amount of fully processed collagen into a matrix (Chen et al., 2009), leading to an excess of unprocessed procollagen in the culture media. Our results indicate that tardiness is due to a lack of sufficient co-factors for cleavage. However, it could also be affected by slow BMP-1 activation, which is dependent on pro-BMP-1 cleavage in the trans-Golgi network by furin-like/paired basic proprotein convertases (Leighton and Kadler, 2003).

Taken together, our analyses of procollagen processing in solution are consistent with heparin/HS behaving as both a crowding molecule and a platform for binding multiple BMP-1 and procollagen molecules. This leads us to our hypothesis that heparin
promotes procollagen processing by increasing the local concentration of BMP-1 and by acting as a linker to bridge BMP-1 with its binding site on FN.

**Processing in the ECM**

As the FN matrix is a dense, fibrillar network, it can act as a biological reservoir concentrating the proteins and metalloproteases that interact with collagen and regulate its processing and assembly. 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 (Figure 5.2-2). 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 on FN through conformational changes of FN domains. Our results do not distinguish between these mechanisms and, in fact, these mechanisms are not mutually exclusive. The positions of binding sites on FN for BMP-1 and heparin and for collagen and the locations of binding sites on collagen for FN suggest the intriguing idea that FN fibrils are not simply accumulating the components in the matrix but might also be providing a template for alignment of procollagens along FN fibrils to facilitate collagen-collagen interactions and fibrillogenesis (Figure 5.2-2, inset).

It appears that multi-molecular 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.
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 heparan sulfate) are added together to the matrix. Inset: Example of procollagen and FN alignment to facilitate processing. The FN-binding site on procollagen aligns with the collagen/gelatin-binding domain (GBD) on FN. This positions the HepII domain of FN adjacent to the cleavage site of the C-propeptide of procollagen. This diagram is the same as in Figure 3.2-8.
Chapter 5.3 – Future Directions

BMP-1 activity is enhanced by the protein PCPE-1, although a consensus on the mechanism of stimulation has not been reached. It has been proposed that interactions with cell-surface HSPGs may play a role in enhancement of BMP-1 by PCPE-1 (Weiss et al., 2010). As heparin can also bind PCPE-1 (Bekhouche et al., 2010), and since PCPE-1 can bind FN, heparin may be acting as a super-stimulator by bringing together BMP-1 and its enhancer and bringing that complex to FN where BMP-1 can bind and increase its activity further (Huang et al., 2009). Since all of these proteins—FN, procollagen, BMP-1, and PCPE-1—bind heparin, a FN-independent processing mechanism could be playing out based on complexing with heparin.

We speculate that FN binding may regulate the ability of PCPE-1 to enhance BMP-1 activity. This question could be addressed using the system described here combined with PCPE-1 specific reagents. Similarly, LOX has been shown to bind FN (Fogelgren et al., 2005), which may augment its intermolecular collagen cross-linking activity. Furthermore, LOX is secreted as a pro-enzyme that requires activation by BMP-1 (Lucero and Kagan, 2006). Complex formation may be required to activate LOX and initiate cross-linking of processed collagen molecules adjacent to FN, and BMP-1 and/or FN may regulate LOX activity even after activation. Whether FN coordinates all of these activities during collagen fibrillogenesis remains to be determined.

Examining how the N-propeptide proteinase ADAMTS-2 is regulated, and if FN matrix and/or heparin/HS affects activity, is necessary as well. There is limited knowledge as to whether ADAMTS-2 can bind to heparin/HS, or even FN. However, the C-terminal domain of ADAMTS-2 was determined to have homology with the second
properdin repeat of thrombospondin-1, which is known to bind heparin and promote cell adhesion (Prockop et al., 1998). Other than its role in N-propeptide cleavage, how its N-propeptide proteinase activity is regulated is unknown. Determining binding partners of ADAMTS-2 and cross-referencing them with FN would provide preliminary data that may suggest a regulatory mechanism similar to that of BMP-1, with respect to heparin/HS and FN.

By examining how procollagen propeptide proteinases are regulated, and how collagen-related molecules interact with the FN matrix, we can develop our understanding of the basis for fibrosis and connective tissue diseases. This may lead to the development of new therapies that target propeptide proteinases, FN binding, GAG associations, or any number of regulatory components that are the causal element of ECM-based diseases.
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