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Targeting CCN Proteins in the Treatment of Dermal Fibrosis

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Abstract

Fibrotic disorders, including the inflammatory connective tissue disease systemic sclerosis (SSc), contribute to 45% of deaths in the Western world. Currently there is no universally agreed-upon treatment for fibrosis. The CCN family of matricellular proteins are tightly spatiotemporally regulated proteins involved in development and wound healing, and are aberrantly expressed in fibrotic disease, including in SSc. CCN1 and CCN2 are overexpressed in SSc and contribute to the progression of animal models of fibrosis. CCN3 is reciprocally regulated to CCN1 and CCN2 and has been shown to suppress CCN2-mediated fibrogenic activity in kidney fibrosis. This led to the development of therapeutic peptides based on CCN3 (BLR-200), which have anti-fibrotic activity *in vitro*. The exact mechanisms of CCN protein activity in dermal fibrosis, including the anti-fibrotic ability of CCN3, have not yet been elucidated. Given this, I tested the hypothesis that CCN protein dysregulation permits a pro-fibrotic microenvironment, leading to increased myofibroblast activation and collagen deposition, that can be prevented by treatment with CCN3-based peptides. First, I used cell culture models to investigate CCN regulation in dermal fibroblasts and found that CCN1 and CCN2 were reciprocally expressed to CCN3 through divergent non-canonical TGFβ signaling pathways. Moreover, in cell culture, CCN3 overexpression inhibited CCN2 protein expression in dermal fibroblasts. Second, I used a murine model of bleomycin-induced SSc dermal fibrosis to assess the ability of BLR-200 to prevent fibrosis and impair early fibroblast response to a pro-inflammatory microenvironment. I found that BLR-200 prevented fibrosis in bleomycin-induced SSc fibrosis, as indicated by impairment of skin thickening, collagen deposition, and myofibroblast activation. Furthermore, BLR-200 treatment prevented bleomycin-induced CCN1 and CCN2 expression. Finally, through single-cell RNA-sequencing analysis, I found that BLR-200 impaired the ability of collagen-expressing fibroblasts to respond to bleomycin-induced inflammatory-driven fibrosis. BLR-200 prevented overexpression of pro-inflammatory genes including Il6, Cxcl2, and NLRP3 inflammasome markers. The results presented here suggest that CCN proteins play an important role in dermal fibrosis. Targeting the pro-fibrotic activity of CCN1 and CCN2 using endogenously derived CCN3-based peptides can prevent multiple pro-fibrotic changes and represents a novel therapeutic approach for treatment of SSc fibrosis.

Keywords: fibrosis, inflammation, systemic sclerosis, dermis, myofibroblast, fibroblast, matricellular protein, CCN1, CCN2, CCN3, bleomycin-induced fibrosis, single-cell RNA-sequencing

Lay Summary

Fibrotic diseases such as systemic sclerosis (scleroderma; SSc) currently have no therapy. These diseases are characterized by extensive scar tissue deposition (largely comprised of collagen), resulting in tissue dysfunction, organ failure, and often death. Patients with SSc will often experience severe scar tissue formation in their skin and internal organs, leading to a significant decrease in quality of life. In the scar tissue, excessive mechanical tension is responsible for the maintenance of the scar. The cells within scar tissue, known as fibroblasts, contribute to this mechanical tension and are essential for fibrosis. A group of proteins called Cellular Communication Network (CCN) proteins are also involved in maintenance of this scar tissue. Two members of this group, CCN1 and CCN2, are expressed in scar tissue and promote the mechanical tension that drives scar tissue formation. A related member of the group, CCN3, is not found in scar tissue, and might act to inhibit the effects of CCN1 and CCN2. In my thesis, I investigate this group of proteins in skin fibrosis, and test whether therapeutic treatment based on CCN3 can block skin fibrosis in mice. First, I found that CCN3 inhibited CCN2 in skin fibroblasts, indicating the potential of CCN3 to prevent the fibrotic activity of CCN2 in skin fibrosis. Second, I found that CCN3-based treatment prevented excessive scar tissue deposition and all the general features of skin fibrosis in mice. Furthermore, CCN3-based treatment inhibited CCN1 and CCN2 in these mice. I also found that CCN3-based treatment impaired the ability of fibroblasts to contribute to the fibrosis. Overall, my thesis contributes to our knowledge of the role of CCN proteins in dermal fibrosis. These results also emphasize the therapeutic potential of CCN3 and CCN3-based therapies for the treatment of skin fibrosis in SSc and potentially other fibrotic diseases.

Co-Authorship Statement

Chapter 2 constitutes a manuscript that has been accepted to a peer-reviewed journal, chapter 3 and chapter 4 will contribute to the future publication of a manuscript. Contributions of co-authors to each chapter are as follows:

Chapter 2:

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AL and AP conceptualized the study. All experiments were performed by AP. BP contributed to methodology by gifting us with unique antibodies to detect CCN3. The manuscript was written by AL, BP, and AP.

Chapter 3:

Authors: Peidl A, Riser BL, Quesnel K, Aslam N, Varani J, Leask A.

AL, BLR, and AP conceptualized the study. BLR provided therapeutic peptides based on CCN3. AP maintained mouse lines and performed all animal experiments with assistance from KQ. NA and JV are collaborators from University of Michigan who facilitated proteomic studies and assisted with proteomic analysis. AP performed all other experimental analysis. AP wrote the manuscript with revisions from AL and BLR.

Chapter 4:

Authors: Peidl A, Riser BL, Aslam N, Varani J, Gurbaksh B, Leask A.

AL, BLR, and AP conceptualized the study. BLR provided therapeutic peptides based on CCN3. AP maintained mouse lines and performed all animal experiments. NA and JV facilitated proteomic studies and assisted with proteomic analysis. BG is a collaborator from University of Toronto who performed single-cell RNA-sequencing. AP performed all other experimental analysis. AP wrote the manuscript with revisions from AL and BLR.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACTB	Beta actin
ACTA2	Actin alpha 2, smooth muscle (Alpha smooth muscle actin)
AKT	Protein kinase B family
ALK5	Activin receptor like kinase 5 (Transforming growth factor beta receptor 1)
AP1	Activator protein 1
BLM	Bleomycin
CCN	Cellular communication network
cDNA	Complementary DNA
COL1A2	Collagen type I alpha 2 chain
CSF	Colony stimulating factor
CT	C-Terminal
CXCL	C-X-C motif chemokine ligand
DAB	3,3′-Diaminobenzidine
dsSSc	Diffuse cutaneous systemic sclerosis
ECM	Extracellular matrix
EDN1	Endothelin 1
EGR1	Early growth response 1
ERK	Mitogen-activated protein kinase 1
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase (Protein tyrosine kinase 2)
FAP	Fibro-adipogenic progenitor

HSPG	Heparan sulfate proteoglycan
IGFBP	Insulin like growth factor binding protein
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
ITGA11	Integrin subunit alpha 11
lcSSc	Limited cutaneous systemic sclerosis
LOX	Lysyl oxidase
MEK1	Mitogen-activated protein kinase kinase 1
MGP	Matrix Gla protein
mRNA	Messenger ribonucleic acid
OSR2	Odd-skipped related transcription factor 2
P4HA1	Prolyl 4-hydroxylase subunit alpha 1
PAI-1	Plasminogen activator inhibitor 1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
qPCR	Quantitative real-time polymerase chain reaction
RAC1	Ras-related C3 botulinum toxin substrate 1
Rna18s	RNA 18s ribosomal 5
scRNA-seq	Single-cell RNA-sequencing
SFRP2	Secreted frizzled related protein 2
SOX2	SRY-box transcription factor 2
SPARCL1	Secreted protein acidic and cysteine rich like 1
SRC	Sarcoma proto-oncogene, non-receptor tyrosine kinase
SSc	Systemic Sclerosis

TAK1	Mitogen-activated protein kinase kinase kinase 7
TAZ	Transcriptional coactivator with PDZ-binding motif
TEK	TEK receptor tyrosine kinase, endothelial
TGFβ	Transforming growth factor beta
TSP	Thrombospondin
VWC	Von Willebrand Factor C Domain Containing
WNT10B	Wnt (Wingless-type MMTV integration site) family member 10B
YAP1	Yes associated protein 1
αSMA	Alpha smooth muscle actin

Chapter 1

1 Introduction

Approximately 45% of the deaths in the developed world result from diseases that have a fibrotic component (Borthwick et al., 2013). However, there are currently no specific anti-fibrotic therapies approved for clinical use. My thesis advances the concept that targeting CCN proteins, by using a 14 amino acid CCN3-derived peptide, represents a novel, viable anti-fibrotic approach. Thus, the implications of my thesis are of high potential clinical impact.

The extracellular matrix (ECM), an extensive network consisting of a variety of extracellular proteins such as collagens, enzymes, and glycoproteins, is essential for development and structural integrity of tissues, including the skin (Kielty & Shuttleworth, 1997). During tissue repair, in response to inflammation, mesenchymal cells such as fibroblasts proliferate and migrate into the wound where they synthesize, adhere to, and contract new ECM (Singer & Clark, 2008). The specialized activated fibroblasts that execute this function are called myofibroblasts, which are characterized by their highly contractile nature (Gabbiani et al., 1971; Hinz, 2010, 2016). If there is a failure to transition back to normal tissue homeostasis, myofibroblasts persist in the site of injury, resulting in the excessive production and contraction of ECM that characterizes scar tissue (Kendall & Feghali-Bostwick, 2014; Walraven & Hinz, 2018). Excessive scar tissue causes chronic fibrotic disease such as liver cirrhosis, diabetes, pulmonary hypertension, and cardiovascular disease (Wynn, 2008). Fibrosis can affect individual organs, or be systemic, such as in the autoimmune connective disease systemic sclerosis (SSc; scleroderma). In its diffuse form, patients with SSc experience fibrosis of the skin, liver, kidney, pancreas, oral cavity, and lungs (Allanore et al., 2015; Elhai et al., 2015). As such, we anticipate that understanding and characterizing fibrogenesis in SSc will greatly enhance our ability to treat this and other fibrotic conditions.

1.1 Overview of Systemic Sclerosis

SSc is a complex autoimmune disease involving the connective tissues of many organs (Asano, 2018; Elhai et al., 2015; McCray & Mayes, 2015). Although the etiology of SSc is largely unknown, its pathological manifestations generally result from three distinct processes: (1) production of autoantibodies, due to an abnormal innate and adaptive immune response; (2) fibroproliferative vasculopathy; and (3) persistent myofibroblast activation, leading to excessive accumulation of ECM in the skin, blood vessels and the internal organs (Allanore et al., 2015; Cutolo et al., 2019; Varga & Abraham, 2007). It is unlikely that the presence of autoantibodies directly contributes to the pathology of SSc but rather appears to merely be representative of a generally heightened immune response (Stern and Denton, 2015). However, it is generally accepted that vascular dysfunction and fibrosis are the principal drivers of the clinical features of SSc (Stern and Denton, 2015). My thesis specifically focuses on developing strategies to control the skin fibrosis observed in SSc.

In North America, the prevalence of SSc is estimated to be approximately 20-40 cases per 100,000 population; but there may be as many as 40,000 SSc patients in Canada (Thompson & Pope, 2002; Zhong et al., 2019). Worldwide, the annual incidence of SSc ranges from 10.9 cases/million to 43 cases/million (Gabrielli et al., 2009). The exact cause of SSc is unknown, however, it is likely to be initiated when genetically predisposed individuals excessively respond to as-yet-unidentified environmental stimuli (Allanore et al., 2015). Clinical symptoms can range from mild to severe, and include sclerodactyly, loss of mobility, generalized pain, Raynaud's phenomenon, telangiectasia, impaired oral hygiene, and pruritis (Elhai et al., 2015). SSc has two subsets based on the extent of skin involvement: limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc). LcSSc involves localized fibrosis of the skin with minimal systemic involvement, while dcSSc has widespread skin and internal organ involvement (Asano, 2018; Elhai et al., 2015; Leask, 2015). Major organ involvement often leads to high mortality and poor survival; in particular, pulmonary fibrosis and pulmonary arterial hypertension cause more than half of SSc-related health events (Denton et al., 2018).

1.1.1 Fibrosis in Systemic Sclerosis

In patients with SSc, early pathological changes in clinically involved connective tissue include vasculopathy, which can manifest clinically as Raynaud's phenomenon (Cutolo et al., 2019; Manetti et al., 2013; Maurer et al., 2014; Stern and Denton, 2015). Vascular damage and endothelial dysfunction lead to infiltration of immune mediators, including macrophages and T helper cells (Kreuter et al., 2009; Roumm et al., 1984; Taroni et al., 2017). This immune cell infiltration leads to an aberrant local immune response, resulting in the release of several pro-inflammatory and pro-fibrotic cytokines, such as interleukin-1 (IL-1), IL-4, IL-6, IL-13, and transforming growth factor-beta isoforms (TGFβ) (Clark et al., 2015; King et al., 2018). This permits a pro-fibrotic microenvironment, which promotes activation of myofibroblasts within the affected lesion. In addition, epigenetic changes in SSc myofibroblasts have been shown to contribute to their activation. For example, altered patterns of histone acetylation can result in increased expression of pro-fibrotic genes (Wang et al., 2006). Collectively, this results in a persistent activated phenotype of myofibroblasts that leads to pathologic fibrosis in SSc.

1.2 The Myofibroblast

The main effector cells in fibrosis are a type of differentiated, highly contractile fibroblast termed myofibroblasts (Hinz, 2010). Since their original discovery (Gabbiani et al., 1971), considerable progress has been made in understanding their role in normal physiology and pathological conditions. Myofibroblasts characteristically overexpress alpha-smooth muscle actin (αSMA; ACTA2), which is organized into stress fibers that exert mechanical forces on the surrounding ECM through focal adhesions (Desmoulière et al., 1993; Hinz et al., 2017). Myofibroblasts are essential during normal tissue repair, but abnormally persistent in fibrotic disease (Gabbiani, 2003). During wound healing, fibroblasts differentiate into myofibroblasts in response to cytokines such as TGFβ. Once activated, myofibroblasts contribute to wound healing by synthesizing ECM components such as type I collagen and fibronectin, as well as contracting the surrounding environment, facilitating wound closure (Gabbiani et al., 1971; Tomasek et al., 2002). When tissue repair is complete, myofibroblasts will clear from the wound area, via apoptosis or deactivation (Desmoulière et al., 1995; Hinz, 2016). In fibrotic conditions, such as SSc, myofibroblasts

persist, leading to a prolonged increase in deposition and contraction of ECM, ultimately resulting in an abnormally stiff microenvironment (Kissin et al., 2006; Wynn, 2008). This matrix stiffness acts in a feed-forward, autocrine manner to further promote activation of myofibroblasts, leading to persistent, pathological fibrosis (Hinz et al., 2019; Parker et al., 2014).

There is much debate in the field regarding the origin of activated myofibroblasts in pathological fibrosis. However, it is widely agreed-upon that myofibroblasts within fibrotic lesions possess complex cellular heterogeneity. Recent developments in cell-lineage tracing and single-cell RNA-sequencing (scRNA-seq) to detect various cellular markers has shed some light on the contributions of several cell types to this heterogenous population. Tissue resident fibroblasts have been shown to contribute to the activated myofibroblast pool in nearly all injured or fibrotic organs, including the kidney (Asada et al., 2011), liver (Iwaisako et al., 2014), eye (Funderburgh et al., 2003; Marino et al., 2017), heart (Kanisicak et al., 2016), and the skin (Rinkevich et al., 2015; Tsang et al., 2019). In contrast, several reports have identified perivascular progenitor cells as the main contributors to the myofibroblast population in fibrotic tissue (di Carlo & Peduto, 2018). Pericytes, or pericyte-like progenitor cells, were shown to give rise to activated myofibroblasts in models of kidney (Humphreys et al., 2010; Lin et al., 2008), lung (Hung et al., 2013), liver (Mederacke et al., 2013) and skin fibrosis (Dulauroy et al., 2012). Furthermore, fibro-adipogenic progenitors (FAPs) have been shown to contribute to skeletal muscle fibrosis (Joe et al., 2010). Therefore, it is hypothesized that a dominant portion of activated myofibroblasts in fibrotic tissue arise from progenitor cells. Given the complexity of fibrosis, the origin of myofibroblasts may also be dependent on the tissue being examined. Interestingly, there is considerable evidence suggesting that resident tissue fibroblasts exhibit plasticity and can acquire a progenitor cell phenotype capable of forming multiple different sub-types of fibroblasts (Contreras et al., 2019; Takahashi & Yamanaka, 2006; Tsang et al., 2019). For example, resident connective tissue fibroblasts in muscle are phenotypically and biochemically equivalent to stromal FAPs *in vitro* (Contreras et al., 2019). Furthermore, collagen-expressing resident dermal fibroblasts acquire a progenitor-like cell intermediate while differentiating into activated myofibroblasts (Tsang et al., 2019). Thus, while several myofibroblast origins have been

suggested, it is highly likely that plastic tissue resident fibroblasts are the principal contributors to this population, especially in the dermis.

In a murine model of bleomycin-induced dermal fibrosis, an important source of activated myofibroblasts is a collagen-lineage pericyte-like progenitor cell expressing the transcription factor SRY-Box transcription factor 2 (SOX2) (Liu et al., 2014; Tsang et al., 2020). SOX2 is a marker of progenitor cells in the mesoderm and regulates several genes involves in self-renewal and differentiation (Kopp et al., 2008). SOX2-expressing myofibroblasts in bleomycin-induced dermal fibrosis also stain positively for pro-fibrotic markers including αSMA, type I collagen, Cellular communication network factor 2 (CCN2; formerly CTGF) and CCN1 (formerly CYR61) (Tsang & Leask, 2014). Further studies have revealed that these SOX2-expressing myofibroblasts are derived from collagen type I alpha 2 (COL1A2)-expressing fibroblasts, suggesting that type I collagen-expressing cells acquire a progenitor cell-like intermediate in the process of phenotypically switching to persistently activated myofibroblasts (Tsang et al., 2019). These SOX2-expressing progenitors are also required for bleomycin-induced dermal fibrosis (Liu et al., 2013). Thus, it appears that the plasticity of tissue resident fibroblasts plays an important role in dermal fibrogenesis. That said, fibroblasts themselves are heterogeneous, and it remains unclear as to which fibroblast subpopulations contribute to and/or differentiate into myofibroblasts in fibrosis. Indeed, understanding the origin of myofibroblasts and molecular mechanisms leading to persistent myofibroblast activation can give insights into fibrogenesis and provide the basis for novel anti-fibrotic treatments. My thesis aims to identify the collagen-linage fibroblast subpopulations essential for dermal fibrosis.

1.2.1 TGFβ and Adhesive Signaling

The most well-characterized mediator of myofibroblast differentiation is TGFβ. In normal wound healing, TGFβ plays a role in the transition of fibroblasts into myofibroblasts, thus facilitating wound closure (Desmoulière et al., 1993; Kane et al., 1991). In pathological fibrosis, activated TGFβ signaling persists and plays a major role in development of harmful scar tissue in fibrotic lesions (Kissin et al., 2002; Roberts et al., 1986). In SSc, TGFβ expression is dysregulated, and a TGFβ-responsive mRNA signature in myofibroblasts correlates with the severity of fibrosis (Sargent et al., 2010; Sfikakis et al.,

1993). TGFβ mainly elicits its effect on fibroblasts by inducing signaling pathways that result in transcription of genes responsible for cell adhesion, proliferation, and ECM production (Kissin et al., 2002; Malmstrom et al., 2004; Verrecchia & Mauviel, 2002). Canonical TGFβ signaling mediates the early response of fibroblasts to TGFβ through activation of the TGFβ receptor type I (Activin linked kinase 5; ALK5) and downstream activation of the SMAD family of proteins (Extensively reviewed by Massagué, 1998). In fibroblasts, TGFβ-induced activation of the ALK5/SMAD pathway can drive the expression of pro-fibrotic genes and a contractile phenotype (Holmes et al., 2001; Ishida et al., 2006; K. Thompson et al., 2014). However, TGFβ-induced pro-fibrotic gene expression appears to be differentially regulated in myofibroblasts cultured from SSc patients (Holmes et al., 2001). Although inhibition of ALK5 in SSc myofibroblasts results in reduced expression of type 1 collagen, the overexpression of αSMA and pro-fibrotic CCN2 were not inhibited (Chen et al., 2006), suggesting that alternative signaling pathways mediate the effects of TGFβ.

Indeed, it has been well demonstrated that a non-canonical, mechanosensitive, pro-adhesive signaling pathway plays a crucial role in the ability of TGFβ to promote fibrosis (Leask, 2020a). This pathway, mediated through activation of integrins and focal adhesion kinase (FAK), is constitutively active in lesional SSc myofibroblasts (Lagares et al., 2012; Thannickal et al., 2003). Overexpression and over-activation of FAK is also a hallmark of the activated myofibroblast phenotype in SSc; inhibition of FAK suppresses this phenotype, including inhibition of αSMA, CCN2, and contraction ability (Mimura et al., 2005; Shi-Wen et al., 2012). Furthermore, in other cell culture models of fibrosis, inhibition of FAK results in prevention of TGFβ-induced αSMA expression, stress fiber formation and cellular hypertrophy (Kuk et al., 2015; Liu et al., 2007; Murphy-Marshman et al., 2017). Integrin activation at the cell surface leads to FAK phosphorylation in myofibroblasts; activated FAK then forms a complex with Src proto-oncogene non-receptor tyrosine kinase (SRC) and induce a complex signaling cascade involving several pro-adhesive signal transducers including ras-related family small GTPase 1 (RAC1), mitogen-activated protein kinase 1 (extracellular signal-regulated kinase; ERK), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase kinase 1 (MEK1), and mitogen-activated protein kinase kinase kinase 7 (TGFβ-activated kinase 1; TAK1)

(Figure 1-1) (Extensively reviewed by Leask, 2020a). Downstream transcriptional (co)factors of this adhesive signaling pathway include activator protein 1 (AP1), early growth response 1 (EGR1) and the yes-associated protein 1 (YAP1)/transcriptional coactivator with PDZ-binding motif (TAZ) complex (Figure 1-1) (Dupont et al., 2011; Leask, 2020a). The YAP1-TAZ complex is an important sensor and mediator of mechanotransduction, and its transcriptional targets include multiple pro-fibrotic factors including αSMA and CCN2 (Dupont et al., 2011; Leask et al., 2003; Nallet-Staub et al., 2014; Shi-wen et al., 2021), which can feed forward in an autocrine manner, promoting further ECM contraction and mediating the expression of fibrosis-related genes. Notably, YAP1 activation and nuclear localization are observed in lesional SSc fibroblasts, suggesting a potential role in SSc pathogenesis (Toyama et al., 2018). Furthermore, inhibition of YAP1 in dermal fibroblasts *in vitro* results in loss of the persistent myofibroblast phenotype, including ECM contraction and overexpression of αSMA and CCN2 (Shi-wen et al., 2021). Collectively, these data indicate that myofibroblasts possess an intrinsically active autocrine pro-adhesive signaling loop, mediated by FAK/YAP1 signaling, that both responds to and produces mechanical tension within the fibrotic microenvironment, thus contributing to the persistent activated myofibroblast phenotype. Accordingly, targeting this feedback loop represents a potential therapeutic approach for treatment of fibrosis.

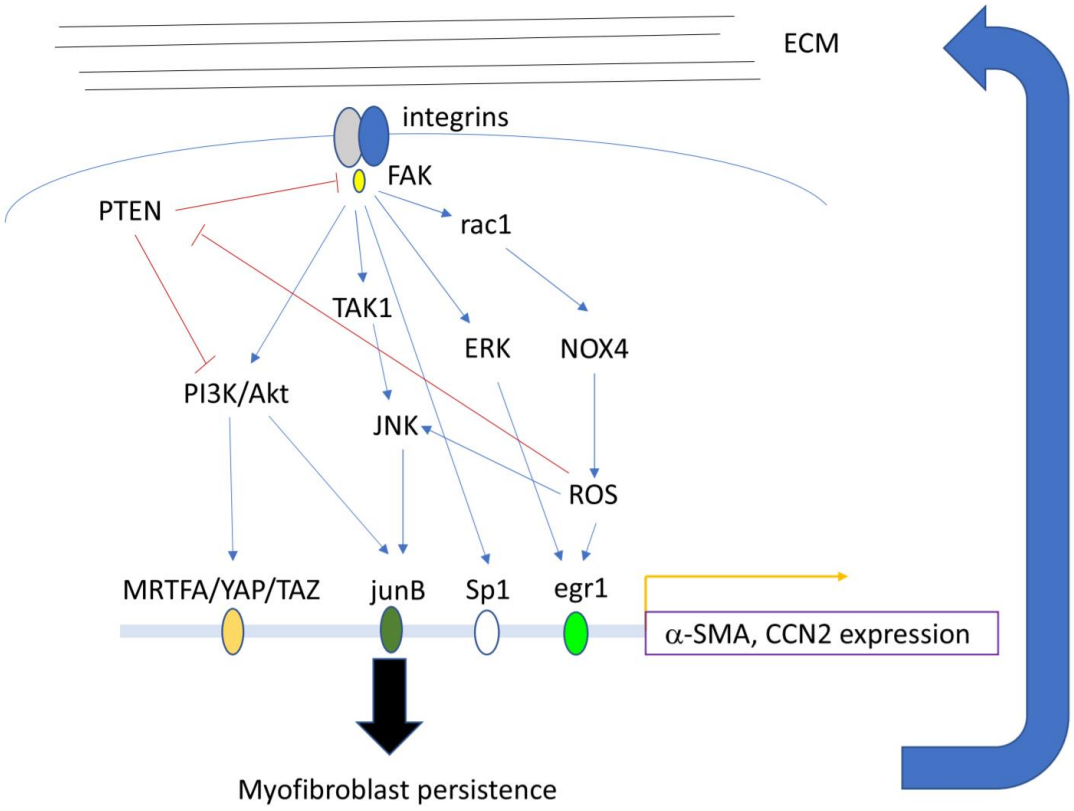

Figure 1-1. Integrated network of pro-adhesive signaling resulting in myofibroblast persistence in SSc.

(Reprinted from *Figure 1. The hard problem: Mechanotransduction perpetuates the myofibroblast phenotype in scleroderma fibrosis*. Leask A. Wound Repair and Regeneration. 2021; 29(4): 582-587)

1.2.2 Therapies Targeting TGFβ and Adhesive Signaling

Given its prominent role in fibrosis, TGFβ has been well-studied as an anti-fibrotic target. Multiple therapeutic strategies targeting TGFβ have progressed into clinical trials for various indications. Metelimumab (CAT-192), an antibody targeting TGFβ1, underwent a phase I/II clinical trial in early dcSSc patients (Clinicaltrials.gov, NCT00043706), but showed no significant efficacy, due to low binding affinity *in vivo* (Denton et al., 2007). Fresolimumab, a monoclonal antibody that targets all TGFβ isoforms, was evaluated in an open-label phase I clinical trial in dcSSc (Clinicaltrials.gov, NCT01284322). Trial results showed that fresolimumab treatment reduced TGFβ-regulated gene expression and had positive outcomes on skin fibrosis (Rice et al., 2015). However, further clinical trials have not yet been initiated, likely due to serious concerns raised by an increased incidence of keratocanthomas and squamous cell carcinomas in patients (Lacouture et al., 2015). The emergence of these skin lesions is likely due to on-target effects of fresolimumab (Morris et al., 2014). In addition, anti-TGFβ treatments are often associated with several safety concerns emerging from off-target effects, due to the highly pleiotropic nature of TGFβ (Györfi et al., 2018). For example, TGFβ exhibits crucial anti-inflammatory effects, and mice with homozygous deletion of TGFβ die 3-4 weeks after birth due to severe systemic inflammation (Shull et al., 1992). Furthermore, serum levels of TGFβ in patients with dcSSc were lower than healthy controls, and inversely correlated with more severe skin fibrosis, suggesting that TGFβ may be sequestered in active involved SSc (Dziadzio et al., 2005). Collectively, these data indicate that a more specific anti-fibrotic should be considered.

Anti-fibrotic treatments aimed at inhibition of FAK/adhesive signaling have shown promising results in animal models (Kinoshita et al., 2013; Lagares et al., 2012). However, FAK inhibitors have shown very little clinical efficacy in anti-fibrotic indications. Since FAK signaling can play a role in several cellular processes, including migration, proliferation, and survival, inhibiting FAK can have deleterious effects on normal physiology, including healthy tissue repair (Dugina et al., 2001; llić et al., 1995; Schaller, 2010). Several integrin-targeting anti-fibrotic strategies have also been tested in clinical trials, with mixed success. Abituzumab, an integrin αv antibody, entered phase I clinical

trials for lung disease in SSc, however the trial was terminated early due to difficulties enrolling suitable study participants (Clinicaltrials.gov, NCT02745145). Further studies have yet to be initiated. BG000111, an integrin αvβ6 antibody, was withdrawn from phase II clinical trials for IPF due to health and safety concerns (Clinicaltrials.gov, NCT03573505). GSK3008348, a small molecule inhibitor of integrin αvβ6, also underwent phase I clinical trials for IPF and showed a relatively good safety profile (Clinicaltrials.gov, NCT03069989). GSK3008348 has shown promising anti-fibrotic effects in animal models, however clinical trials have been temporarily placed on hold (John et al., 2020; Slack et al., 2021). Currently, the most advanced integrin-targeting therapy in clinical trials is the dual integrin αvβ1/αvβ6 inhibitor, PLN-74809, which is currently in phase II for IPF and primary sclerosing cholangitis (PSC) (Clinicaltrials.gov, NCT04396756; NCT04480840). While PLN-74809 has shown promise for treatment of IPF and PSC, targeting integrin β6 in dermal fibrosis may not be as effective due to the important role of integrin β1 in dermal fibroblasts (Liu et al., 2009, 2010). Given the issues with current therapies, targeting specific downstream mediators of adhesive signaling may be a preferable anti-fibrotic strategy.

For example, CCN2, one of six members of the Centralized Communication Network (CCN) family of matricellular proteins, is induced in response to integrin/FAK/YAP1 mediated adhesive signaling, while also playing an important role in activation of integrins at the cell surface (Chen et al., 2001; Graness et al., 2006; Lau, 2016; Shi-wen et al., 2021). Thus, this ‘matricellular’ protein appears to contribute to the autocrine nature of this pro-adhesive signaling loop in myofibroblasts. In fact, studies on the CCN family of matricellular proteins in the context of fibrosis has shown that their dysregulation significantly contributes to fibrogenesis (Liu et al., 2011; Quesnel et al., 2019). Therefore, CCN proteins may provide a more specific target on which to base an anti-fibrotic therapy.

1.3 Matricellular Proteins

Matricellular proteins are a group of adhesive proteins secreted into the ECM (Chiodoni et al., 2010). They do not contribute to the structural elements within the microenvironment; however, they have extensive function in mediating the dynamic biochemical and mechanical changes of the ECM to surrounding cell populations (Bornstein, 2009). This

function is achieved by their ability to interact with cell-surface receptors, cytokines, growth factors, hormones, and structural components of the ECM (Bornstein, 2006; Bradshaw, 2016). The expression of matricellular proteins is highly spatially and temporally regulated. Their expression is higher in developing tissue but is generally low in adult tissue (Bradshaw, 2016). However, tissue response to injury results in robust overexpression of particular matricellular proteins, where they mediate a wide variety of critical biological processes including ECM organization, inflammation, cell migration, differentiation, wound healing, and fibrosis (Bornstein, 2009; Chiodoni et al., 2010; Prakoura & Chatziantoniou, 2017). Additionally, the contribution of dysregulated matricellular proteins to fibrogenesis in SSc has been well-established (Feng & Gerarduzzi, 2020). Since matricellular proteins often have local and context-specific effects on the surrounding ECM, they represent an ideal specific target for drug intervention.

1.4 Centralized Communication Network Family

The CCN family of matricellular proteins is a group of six secreted proteins that show expression patterns that are dysregulated in connective tissue pathologies, including in SSc (Leask & Abraham, 2006). The most prominent members of the family, CCN1, CCN2, and CCN3 (formerly NOV), were originally discovered in the early 1990s (Bradham et al., 1991; Joliot et al., 1992; O'Brien et al., 1990; Perbal et al., 2018). Since their discovery, extensive research has been done to understand the complex range of CCN protein activity. The structure of CCN proteins includes four distinct conserved modules: an insulin-like growth factor binding protein (IGFBP) domain, a Von Willebrand factor (VWC) domain, a thrombospondin (TSP)-like domain, and a cysteine knot C-terminal (CT) domain (Holbourn et al., 2008). Each of these regions has been shown to bind a number of different cell surface receptors, including integrins and heparin-sulfate-proteoglycans (HSPGs) such as syndecan 4 and perlecan (Nishida et al., 2003; Todorovicç et al., 2005). Depending on the specific situation, CCNs have been shown to bind and mediate signaling through a wide variety of integrins including: $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha IIb\beta_3$, $\alpha M\beta_2$, and $\alpha D\beta_2$ (Extensively reviewed by Lau, 2016). Although several integrin binding domains have been identified on CCN proteins, they do not contain the canonical integrin-binding RGD-sequence (Lau, 2016). Additional CCN binding partners include components of the ECM such as

fibronectin, and growth factors such as TGFβ (Abreu et al., 2002; Chen et al., 2004; Hoshijima et al., 2006). Thus, the suspected role of CCN proteins is to integrate and modulate cellular responses to the local environment. Depending on the composition of the microenvironment, for example, the presence of proteins and receptors with which they interact, CCN proteins can mediate a range of biological functions. They have been shown to contribute to cell adhesion, migration, proliferation, differentiation, apoptosis, survival, and senescence (Blom et al., 2001; Chen et al., 2000; Ellis et al., 2003; Feng et al., 2019; Gao & Brigstock, 2004; Grotendorst & Duncan, 2005; Jia et al., 2017; Jun & Lau, 2017; Kireeva et al., 1996; Lafont et al., 2009; Latinkic et al., 2003; Lin et al., 2005; Sakamoto et al., 2002; Su et al., 2020; Todorovicç et al., 2005). Due to their often-subtle, context-dependent effects, the exact mechanisms by which CCN proteins function in pathological conditions such as fibrosis are still unclear, despite extensive studies. Nonetheless, we do know that CCN1, CCN2, and CCN3 are spatiotemporally regulated during tissue repair and dysregulated during fibrosis (Igarashi et al., 1996; Quesnel et al., 2019; Riser et al., 2009; Rittié et al., 2011). Thus, studying this dysregulation may provide insight into mechanisms of fibrosis, and targeting these CCN proteins may represent a novel therapeutic approach.

1.4.1 CCN2: A *Bona Fide* Therapeutic Target in Fibrosis

The most well-studied of the CCN family is CCN2, especially in the context of fibrosis. While CCN2 is essentially absent from healthy adult tissue, it is potently induced by TGFβ and overexpression of CCN2 is a hallmark of fibrosis in nearly all fibrotic conditions, including SSc, IPF, PSC, muscular dystrophy, diabetic nephropathy, myocardial fibrosis, atherosclerosis, gingival hyperplasia, and hepatic fibrosis (Gressner et al., 2006; Igarashi et al., 1996; Koitabashi et al., 2008; Nguyen et al., 2006; Sato et al., 2000; Sun et al., 2008; Yao et al., 2017). In fibroblasts, TGFβ induces CCN2 expression through the canonical ALK5/SMAD pathway, as well as through the non-canonical pro-adhesive signaling pathways mediated by FAK/YAP1 (Mori et al., 1999). CCN2 is a prototypical target of YAP1, and its expression is highly responsive to mechanotransduction (Shi-wen et al., 2021). In pathological conditions, such as SSc, pro-adhesive signaling is required for constitutive CCN2 upregulation; whereas inhibition the ALK5/SMAD pathway in

fibroblasts does not appreciably affect CCN2 expression (Chen et al., 2006; Holmes et al., 2001, 2003; Ponticos et al., 2009; Shi-wen et al., 2009). CCN2 likely plays its role in fibrosis by creating a microenvironment permissive for induction of an aggressive fibrotic response. This is evidenced by previous studies showing that co-injection of CCN2 with TGFβ results in a sustained fibrotic response in mice, whereas TGFβ alone only causes a transient fibrotic response (Mori et al., 1999; Yanagihara et al., 2020). It should also be noted that CCN2 does not mediate the downstream effects of TGFβ, but rather modulates and amplifies the ability of TGFβ to promote fibrosis (Leask, 2020b). This is also supported by the observation that loss of CCN2 expression in fibroblasts does not impair normal cutaneous wound healing (Thompson, et al., 2014). Therefore, the effects of CCN2 seem to be specific to fibrotic conditions.

Many studies have shown that anti-CCN2 strategies are effective in blocking fibrogenesis, including in the kidney, lung, heart, liver, muscle, and skin (Barbe et al., 2020; George & Tsutsumi, 2007; Hong et al., 2018; Makino et al., 2017; Phanish et al., 2010; Ponticos et al., 2009). Genetic analysis using animal models has revealed several context- and cell-specific effects of CCN2 in fibrosis. In a mouse model of muscular dystrophy, conditional knock-out of CCN2 from myofibers resulted in protection from the dystrophic phenotype (Petrosino et al., 2019). CCN2 expression is also required in mouse models of hepatic fibrosis (Pi et al., 2015). Our lab has shown that fibroblast-specific CCN2 expression is required for fibrogenesis in the bleomycin-induced murine model of SSc dermal fibrosis. In these experiments, conditional knockout of CCN2 in collagen-expressing fibroblasts was sufficient to prevent bleomycin-induced increase in skin thickness, collagen deposition, and myofibroblast activation (Liu et al., 2011). Our lab has also shown that CCN2 expression by fibroblasts is required for skin and lung fibrosis caused by overactivation of adhesive/Akt signaling (Liu et al., 2013; Parapuram et al., 2011, 2015). Moreover, in dermal fibrosis, CCN2 is required for the differentiation of resident fibroblasts into activated myofibroblasts, through a SOX2-expressing progenitor cell intermediate (Tsang et al., 2019). Thus, in addition to modulating the fibrotic effects of TGFβ, CCN2 appears to play an important role in mediating the differentiation of progenitor cell intermediates into persistently active myofibroblasts (Hutchenreuther & Leask, 2016; Tsang et al., 2019)

1.4.2 FG-3019

Despite the evidence that CCN2 may represent a viable anti-fibrotic target, there is currently no approved therapy targeting CCN2. However, FG-3019, a humanized monoclonal antibody targeting CCN2, has recently shown promise in animal models and clinical trials (Brenner et al., 2016). FG-3019 has been shown to effectively block metastasis in mouse models of pancreatic cancer, as well as prevent fibrosis in mouse models of IPF, skeletal muscle fibrosis, and SSc (Barbe et al., 2020; Bickelhaupt et al., 2017; Makino et al., 2017; Sakai et al., 2017). FG-3019 is currently in phase III clinical trials for IPF (Clinicaltrials.gov, NCT04419558), Duchene muscular dystrophy (Clinicaltrials.gov, NCT04632940), and pancreatic cancer (Clinicaltrials.gov, NCT03941093). Moreover, in phase II clinical trials for IPF, FG-3019 was shown to be effective in reducing fibrotic biomarkers, had positive outcomes on pulmonary function, and reduced the extent of pulmonary fibrosis in SSc patients with IPF (Raghu et al., 2016). The antibody also underwent phase I/II clinical trials for diabetic nephropathy (Clinicaltrials.gov, NCT00913393), and showed promising initial results (Adler et al., 2010). However, these studies did not progress into phase III (or were not reported), which could be due to lack of efficacy because of issues achieving antibody tissue penetration into target regions. It still remains unclear as to how FG-3019 actually works; however, it may function by clearing CCN2 from tissues into the circulation (Brenner et al., 2016). Evidence also suggests that FG-3019, due to target-mediated elimination, has unusually rapid clearance and short half-life (Brenner et al., 2016). Thus, administration of higher and more frequent doses is likely necessary to achieve biological efficacy. Although FG-3019 represents an exciting advancement in the field of CCN biology, other therapeutic approaches should continue to be explored.

1.4.3 CCN1 in Fibrosis

CCN1 is the second-most studied of the CCN family. In cell culture, CCN1 is induced in fibroblasts by TGFβ in a manner dependent on pro-adhesive FAK/YAP1 signaling pathways, sharing this regulatory pathway with CCN2 (Kuk et al., 2015; Murphy-Marshman et al., 2017; Shi-wen et al., 2021; Thompson et al., 2014). CCN1 also shares a similar modular structure with CCN2, and both proteins have been found to interact with

many of the same integrins and cell-surface receptors, through which they can modulate adhesive signaling in fibroblasts (Chaqour, 2020; Chen et al., 2001; Lau, 2016). Although CCN1 likely represents an anti-fibrotic target, studies examining the exact role of CCN1 in fibrotic models have yielded differing results. This is likely due to the context-specific effects of CCN proteins in general. Thus, the role of CCN1 will depend on the presence of the specific integrins and cell surface receptors with which it interacts. CCN1 expression is increased in inflammatory conditions and fibrosis, including IPF and SSc (Gardner et al., 2006; Grazioli et al., 2015; Krupska et al., 2015; Lau, 2011). In SSc, CCN1 has been shown to play a role in the early inflammatory stages of the disease, evidenced by CCN1 overexpression in dermal fibroblasts from the fibrotic lesions of patients with early onset dcSSc (Gardner et al., 2006; Quesnel et al., 2019). It has also been shown that CCN1 plays a pro-inflammatory role in the pathogenesis of psoriasis by promoting expression of immune mediators and subsequent keratinocyte activation (Sun et al., 2015, 2017). Conversely, CCN1 has also been reported to have anti-fibrotic effects in liver fibrosis, and overexpression of CCN1 in portal myofibroblasts suppressed the extent of fibrogenesis in the liver (Borkham-Kamphorst et al., 2018; Kim et al., 2013). One reason for these effects could be that CCN1 overexpression induces a potent unfolded protein response leading to apoptosis of myofibroblasts, therefore limiting excessive collagen deposition in this model (Borkham-Kamphorst et al., 2016).

Our lab has previously shown that CCN1 expression by fibroblasts is required for development of bleomycin-induced dermal fibrosis (Quesnel et al., 2019). In this study, loss of CCN1 expression in collagen-expressing dermal fibroblasts resulted in impaired collagen alignment and decreased mRNA expression of the collagen cross-linking genes prolyl-4-hydroxylase (*P4ha1*), lysyl oxidase (*Lox*) and procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 (*Plod2*) (Quesnel et al., 2019). This led to the hypothesis that CCN1 contributes to fibrosis by increasing the organization and stability of fibrillar collagen within the ECM. In another study, blockade of CCN1 expression was also shown to prevent inflammatory fibrosis in a model of renal fibrosis (Lai et al., 2013, 2014). Although there are overlapping pro-fibrotic functions between CCN1 and CCN2, it appears that both have distinct roles in pathological fibrosis. Therefore, a useful anti-fibrotic therapy may aim at blocking both CCN1 and CCN2.

1.4.4 CCN3: A Potential Anti-Fibrotic Therapy

CCN3 has been studied much less than CCN1 and CCN2 but has been observed to have unique effects in pathological conditions. In initial reports, CCN3 treatment had inhibitory effects on proliferation of chicken embryonic fibroblasts, while CCN1 and CCN2 had proliferative effects (Perbal, 2001). This provided the first evidence that CCN proteins may be differentially regulated and that CCN3 may act as a negative regulator of the pathological effects of other CCNs.

Indeed, many other studies have confirmed that CCN3 has opposing effects to CCN1 and CCN2 *in vitro* and *in vivo* (Jun & Lau, 2011). In mouse cartilage, CCN3 and CCN2 are reciprocally regulated and have opposing effects on chondrocyte differentiation (Kawaki et al., 2008). CCN3 also has inhibitory effects on vascular smooth muscle cell migration, while CCN1 and CCN2 have stimulatory effects (Shimoyama et al., 2010). Moreover, CCN3 has anti-proliferative effects on mesangial cells and fibroblasts, directly opposing the proliferative effects of CCN1 and CCN2 (Ren et al., 2014; van Roeyen et al., 2008). It has also been reported that CCN3 opposes the tumour promoting effects of CCN1 in multiple models of cancer (Bleau et al., 2005). In the context of fibrosis, CCN3 has been shown to exhibit anti-fibrotic properties and directly opposes the pro-fibrotic effects of CCN1 and CCN2 (Lemaire et al., 2010). In mouse embryonic fibroblasts, CCN3 overexpression results in repression of TGFβ-induced CCN2 mRNA expression, as well as pro-fibrotic markers including αSMA (Abd El Kader et al., 2012; Lemaire et al., 2010). CCN3 inhibition in hepatic stellate cells results in increased expression of CCN2 and other fibrogenic markers including fibronectin, αSMA and type I collagen (Borkham-Kamphorst et al., 2012). In this study, it was found that adenoviral overexpression of CCN2 suppressed CCN3 expression (Borkham-Kamphorst et al., 2012). In addition, CCN3 expression has antagonistic effects on TGFβ-mediated fibrogenic matrix assembly in a cell culture model of SSc (Lemaire et al., 2010).

The role of CCN3 in models of kidney fibrosis has also been extensively studied. In an *in vitro* model of diabetic renal fibrosis, CCN3 and CCN2 work in a yin/yang-like manner to regulate fibrosis development. Addition of CCN3 in this model prevented TGFβ-induced pro-fibrotic gene expression and phenotypic changes (Riser et al., 2009). In response to

TGFβ treatment, mesangial cells increase expression of CCN2, while suppressing CCN3 expression (Riser et al., 2010). In a mouse model of diabetic nephropathy, treatment with recombinant human CCN3 (rhCCN3) was able to prevent markers of primary and secondary renal fibrosis gene activation including CCN2, type I collagen, and plasminogen activator inhibitor-1 (PAI-1). In this study, rhCCN3 treatment also prevented mesangial matrix expansion, podocyte loss, and glomerular hypertrophy, as well as attenuated normal renal function (Riser et al., 2014). Collectively, there is abundant evidence that CCN3 may act as an endogenous antagonist of the pro-fibrotic activity of CCN1 and CCN2.

1.4.5 BLR-200: Therapeutic Peptides Based on CCN3

Building on the observed anti-fibrotic role of endogenous CCN3, a small peptide based on CCN3 (BLR-200) was developed by BLR Bio, as a potential therapy to treat fibrotic disease (Riser et al., 2015). BLR-200 is a 14 amino acid peptide that is based on a specific sequence of endogenous CCN3, with proprietary modifications to increase stability. According to an issued patent, BLR-200 blocks mesangial cell adhesion to CCN2 and inhibits the ability of pro-fibrotic agents to provoke collagen expression in dermal fibroblasts *in vitro* (U.S. Patent No. 9114112B2, 2015). In addition, BLR-200 was able to prevent fibrosis and promote favourable matrix remodeling of the tumour microenvironment in a murine model of orthotopic pancreatic ductal carcinoma (Resoyi et al., 2020).

The use of therapeutic peptides to treat fibrotic diseases, such as SSc, has several important advantages over other potential anti-fibrotic therapies. Currently, there is considerable attention on the use of antibodies, such as FG-3019, to treat fibrotic conditions. A major problem with antibody treatment is poor tissue distribution and limited ability to penetrate solid tissues (Epenetos et al., 1986), such as excessively fibrotic skin (Piersma et al., 2020). The use of small, stable peptides circumvents this issue. In preliminary pharmacokinetic studies, intraperitoneal injection of BLR-200 results in biologically relevant tissue distribution to the pancreas and kidneys (Andrew Leask and Bruce Riser, unpublished data). Antibody treatments also carry the inherent risk of side effects due to immunogenicity (Harding et al., 2010). This could pose an even bigger risk in SSc patients, who experience dysregulated autoimmunity (Stern and Denton, 2015). Since BLR-200 is

designed based on an endogenously expressed protein, the risk of off-target side effects is relatively low. Indeed, toxicity studies have shown that high doses of BLR-200 are well tolerated by mice, with no observable adverse effects (Andrew Leask and Bruce Riser, unpublished data). In addition, therapeutic peptides are easier to synthesize than antibodies, and are therefore less expensive to produce (Marqus et al., 2017). This could have a significant financial impact for patients seeking treatment. Overall, BLR-200 represents a promising novel therapeutic treatment for fibrotic disorders that involve CCN protein dysregulation, including SSc. My thesis aims to determine the specific anti-fibrotic effects of BLR-200 in an animal model of SSc dermal fibrosis.

1.5 Objective and Hypothesis

Since their initial discovery in the 1990s, CCN proteins have been studied extensively. However, due to their complex context-dependent effects and ability to interact with a myriad of proteins, the exact mechanism of CCN activity in pathological conditions remains unknown (Leask, 2020b). Their activity can differ depending on specific cell type and the composition of the ECM. Thus, they likely have different functions depending on the clinical stage of disease. We do know that CCN1 and CCN2 are highly upregulated during dermal fibrosis and have pro-fibrotic effects (Liu et al., 2011; Quesnel et al., 2019). In contrast, CCN3 has been shown to have anti-fibrotic effects (Riser et al., 2014). However, it is unclear how this yin/yang-like relationship is regulated and contributes to fibrosis. Further research is required to determine the exact pathways governing the regulation of CCN proteins, especially in the context of fibrosis. Moreover, *in vivo* studies are required to evaluate the exact biological roles of CCN proteins, since cell culture models are unable to truly recapitulate the intricate complexities of the ECM. Thus, to assess the anti-fibrotic effects of CCN3, animal models are required. The aim of this thesis is to address these gaps in knowledge. Based on the available data, the overarching hypothesis of this thesis is that CCN protein dysregulation permits a pro-fibrotic microenvironment, leading to increased dermal ECM deposition and myofibroblast activation, that can be prevented by treatment with CCN3-based peptides.

1.6 References

Abd El Kader, T., Kubota, S., Janune, D., Nishida, T., Hattori, T., Aoyama, E., Perbal, B., Kuboki, T., & Takigawa, M. (2012). Anti-fibrotic effect of CCN3 accompanied by altered gene expression profile of the CCN family. *Journal of Cell Communication and Signaling*, *7*(1), 11–18. https://doi.org/10.1007/S12079-012-0180-4

Abreu, J. G., Ketpura, N. I., Reversade, B., & Robertis, E. M. de. (2002). Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-β. *Nature Cell Biology*, *4*(8), 604. https://doi.org/10.1038/NCB826

Adler, S. G., Schwartz, S., Williams, M. E., Arauz-Pacheco, C., Bolton, W. K., Lee, T., Li, D., Neff, T. B., Urquilla, P. R., & Sewell, K. L. (2010). Phase 1 Study of Anti-CTGF Monoclonal Antibody in Patients with Diabetes and Microalbuminuria. *Clinical Journal of the American Society of Nephrology: CJASN*, *5*(8), 1428. https://doi.org/10.2215/CJN.09321209

Allanore, Y., Simms, R., Distler, O., Trojanowska, M., Pope, J., Denton, C. P., & Varga, J. (2015). Systemic sclerosis. *Nature Reviews Disease Primers*, *1*(15002), 1–21. https://doi.org/10.1038/nrdp.2015.2

Asada, N., Takase, M., Nakamura, J., Oguchi, A., Asada, M., Suzuki, N., Yamamura, K., Nagoshi, N., Shibata, S., Rao, T. N., Fehling, H. J., Fukatsu, A., Minegishi, N., Kita, T., Kimura, T., Okano, H., Yamamoto, M., & Yanagita, M. (2011). Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice. *Journal of Clinical Investigation*, *121*(10), 3981–3990. https://doi.org/10.1172/JCI57301

Asano, Y. (2018). Systemic sclerosis. *Journal of Dermatology*. https://doi.org/10.1111/1346-8138.14153

Barbe, M. F., Hilliard, B. A., Amin, M., Harris, M. Y., Hobson, L. J., Cruz, G. E., & Popoff, S. N. (2020). Blocking CTGF/CCN2 reduces established skeletal muscle fibrosis in a rat model of overuse injury. *FASEB Journal*, *34*, 6554–6569. https://doi.org/10.1096/fj.202000240RR

Bickelhaupt, S., Erbel, C., Timke, C., Wirkner, U., Dadrich, M., Flechsig, P., Tietz, A., Pföhler, J., Gross, W., Peschke, P., Hoeltgen, L., Katus, H. A., Gröne, H.-J., Nicolay, N. H., Saffrich, R., Debus, J., Sternlicht, M. D., Seeley, T. W., Lipson, K. E., & Huber, P. E. (2017). Effects of CTGF Blockade on Attenuation and Reversal of Radiation-Induced Pulmonary Fibrosis. *Journal of the National Cancer Institute*, *109*(8). https://doi.org/10.1093/JNCI/DJW339

Bleau, A. M., Planque, N., & Perbal, B. (2005). CCN proteins and cancer: two to tango. *Frontiers in Bioscience*, *10*, 998–1009. https://doi.org/10.2741/1594

Blom, I. E., van Dijk, A. J., Wieten, L., Duran, K., Ito, Y., Kleij, L., deNichilo, M., Rabelink, T. J., Weening, J. J., Aten, J., & Goldschmeding, R. (2001). In vitro evidence for differential involvement of CTGF, TGFβ, and PDGF-BB in mesangial response to injury. *Nephrology Dialysis Transplantation*, *16*(6), 1139–1148. https://doi.org/10.1093/NDT/16.6.1139

Borkham-Kamphorst, E., Steffen, B. T., van de Leur, E., Haas, U., & Weiskirchen, R. (2018). Portal myofibroblasts are sensitive to CCN-mediated endoplasmic reticulum stress-related apoptosis with potential to attenuate biliary fibrogenesis. *Cellular Signalling*, *51*, 72–85. https://doi.org/10.1016/J.CELLSIG.2018.07.005

Borkham-Kamphorst, E., Steffen, B. T., van de Leur, E., Tihaa, L., Haas, U., Woitok, M. M., Meurer, S. K., & Weiskirchen, R. (2016). Adenoviral CCN gene transfers induce in vitro and in vivo endoplasmic reticulum stress and unfolded protein response. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1863*(11), 2604–2612. https://doi.org/10.1016/J.BBAMCR.2016.07.006

Borkham-Kamphorst, E., van Roeyen, C. R., van de Leur, E., Floege, J., & Weiskirchen, R. (2012). CCN3/NOV small interfering RNA enhances fibrogenic gene expression in primary hepatic stellate cells and cirrhotic fat storing cell line CFSC. *Journal of Cell Communication and Signaling*, *6*(1), 25. https://doi.org/10.1007/S12079-011-0141-3

Bornstein, P. (2006). Extracellular Matrix: Matricellular Proteins. *Encyclopedia of Respiratory Medicine*, 175–183. https://doi.org/10.1016/B0-12-370879-6/00148-4

Bornstein, P. (2009). Matricellular proteins: an overview. *Journal of Cell Communication and Signaling*, *3*, 163–165. https://doi.org/10.1007/s12079-009-0069-z

Borthwick, L. A., Wynn, T. A., & Fisher, A. J. (2013). Cytokine mediated tissue fibrosis. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1832*(7), 1049–1060. https://doi.org/10.1016/j.bbadis.2012.09.014

Bradham, D. M., Igarashi, A., Potter, R. L., & Grotendorst, G. R. (1991). Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *Journal of Cell Biology*, *114*(6), 1294. https://doi.org/10.1083/JCB.114.6.1285

Bradshaw, A. D. (2016). The Extracellular Matrix. *Encyclopedia of Cell Biology*, *2*, 694–703. https://doi.org/10.1016/B978-0-12-394447-4.20067-9

Brenner, M. C., Krzyzanski, W., Chou, J. Z., Signore, P. E., Fung, C. K., Guzman, D., Li, D., Zhang, W., Olsen, D. R., Nguyen, V.-T. L., Koo, C. W., Sternlicht, M. D., & Lipson, K. E. (2016). FG-3019, a Human Monoclonal Antibody Recognizing Connective Tissue Growth Factor, is Subject to Target-Mediated Drug Disposition. *Pharmaceutical Research*, *33*(8), 1849. https://doi.org/10.1007/S11095-016-1918-0

Bruce L. Riser. (2015). *CCN3 AND CCN3 PEPTIDES AND ANALOGS THEREOF FOR THERAPEUTC USES* (Patent No. US 9,114,112 B2).

Chaqour, B. (2020). Caught between a “Rho” and a hard place: are CCN1/CYR61 and CCN2/CTGF the arbiters of microvascular stiffness? *Journal of Cell Communication and Signaling*, *14*(1), 29. https://doi.org/10.1007/S12079-019-00529-3

Chen, C. C., Chen, N., & Lau, L. F. (2001). The Angiogenic Factors Cyr61 and Connective Tissue Growth Factor Induce Adhesive Signaling in Primary Human Skin

Fibroblasts. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M008087200

Chen, N., Chen, C.-C., & Lau, L. F. (2000). Adhesion of Human Skin Fibroblasts to Cyr61 Is Mediated through Integrin α6β1 and Cell Surface Heparan Sulfate Proteoglycans *. *Journal of Biological Chemistry*, *275*(32), 24953–24961. https://doi.org/10.1074/JBC.M003040200

Chen, Y. (2004). CCN2 (Connective Tissue Growth Factor) Promotes Fibroblast Adhesion to Fibronectin. *Molecular Biology of the Cell*. https://doi.org/10.1091/mbc.E04-06-0490

Chen, Y., Shi-Wen, X., Eastwood, M., Black, C. M., Denton, C. P., Leask, A., Abraham, D. J., Xu, S. W., Eastwood, M., Black, C. M., Denton, C. P., Leask, A., & Abraham, D. J. (2006). Contribution of activin receptor-like kinase 5 (transforming growth factor β receptor type I) signaling to the fibrotic phenotype of scleroderma fibroblasts. *Arthritis & Rheumatology*, *54*(4), 1309–1316. https://doi.org/10.1002/art.21725

Chiodoni, C., Colombo, M. P., & Sangaletti, S. (2010). Matricellular proteins: From homeostasis to inflammation, cancer, and metastasis. In *Cancer and Metastasis Reviews*. https://doi.org/10.1007/s10555-010-9221-8

Clark, K. E., Lopez, H., Abdi, B. A., Guerra, S. G., Shiwen, X., Khan, K., Etomi, O., Martin, G. R., Abraham, D. J., Denton, C. P., & Stratton, R. J. (2015). Multiplex cytokine analysis of dermal interstitial blister fluid defines local disease mechanisms in systemic sclerosis. *Arthritis Research & Therapy*, *17*(1). https://doi.org/10.1186/s13075-015-0575-8

Contreras, O., Rossi, F. M., & Brandan, E. (2019). Adherent muscle connective tissue fibroblasts are phenotypically and biochemically equivalent to stromal fibro/adipogenic progenitors. *Matrix Biology Plus*, *2*, 100006. https://doi.org/10.1016/J.MBPLUS.2019.04.003

Cutolo, M., Soldano, S., & Smith, V. (2019). Pathophysiology of systemic sclerosis: current understanding and new insights. *Expert Review of Clinical Immunology*, *15*(7), 753–764. https://doi.org/10.1080/1744666X.2019.1614915

Denton, C. P., Merkel, P. A., Furst, D. E., Khanna, D., Emery, P., Hsu, V. M., Silliman, N., Streisand, J., Powell, J., Åkesson, A., Coppock, J., Hoogen, F. van den, Herrick, A., Mayes, M. D., Veale, D., Haas, J., Ledbetter, S., Korn, J. H., Black, C. M., & Seibold, J. R. (2007). Recombinant human anti–transforming growth factor β1 antibody therapy in systemic sclerosis: A multicenter, randomized, placebo-controlled phase I/II trial of CAT-192. *Arthritis & Rheumatology*, *56*(1). https://doi.org/10.1002/art.22289

Denton, C. P., Wells, A. U., & Coghlan, J. G. (2018). Major lung complications of systemic sclerosis. *Nature Reviews Rheumatology*, *14*(9), 511–527. https://doi.org/10.1038/s41584-018-0062-0

Desmoulière, A., Geinoz, A., Gabbiani, F., & Gabbiani, G. (1993). Transforming growth factor-β 1 induces alpha-smooth muscle actin expression in granulation tissue

myofibroblasts and in quiescent and growing cultured fibroblasts. *Journal of Cell Biology*, *122*(1), 103–111. https://doi.org/10.1083/JCB.122.1.103

Desmoulière, A., Redard, M., Darby, I., & Gabbiani, G. (1995). Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *American Journal of Pathology*, *146*(1), 66. /pmc/articles/PMC1870783/?report=abstract

di Carlo, S. E., & Peduto, L. (2018). The perivascular origin of pathological fibroblasts. *Journal of Clinical Investigation*, *128*(1), 54–63. https://doi.org/10.1172/JCI93558

Dugina, V., Fontao, L., Chaponnier, C., Vasiliev, J., & Gabbiani, G. (2001). Focal adhesion features during myofibroblastic differentiation are controlled by intracellular and extracellular factors. *Journal of Cell Science*, *114*(18), 3285–3296. https://doi.org/10.1111/J.1600-0765.2006.00886.X

Dulauroy, S., di Carlo, S. E., Langa, F., Eberl, G., & Peduto, L. (2012). Lineage tracing and genetic ablation of ADAM12+ perivascular cells identify a major source of profibrotic cells during acute tissue injury. *Nature Medicine*, *18*(8), 1262–1270. https://doi.org/10.1038/nm.2848

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., le Digabel, J., Forcato, M., Bicciato, S., Elvassore, N., & Piccolo, S. (2011). Role of YAP/TAZ in mechanotransduction. *Nature*, *474*(7350), 179–183. https://doi.org/10.1038/nature10137

Dziadzio, M., Smith, R. E., Abraham, D. J., Black, C. M., & Denton, C. P. (2005). Circulating levels of active transforming growth factor β1 are reduced in diffuse cutaneous systemic sclerosis and correlate inversely with the modified Rodnan skin score. *Rheumatology*, *44*(12), 1518–1524. https://doi.org/10.1093/RHEUMATOLOGY/KEI088

Elhai, M., Avouac, J., Kahan, A., & Allanore, Y. (2015). Systemic sclerosis: Recent insights. *Joint Bone Spine*, *82*(3), 148–153. https://doi.org/10.1016/J.JBSPIN.2014.10.010

Ellis, P. D., Metcalfe, J. C., Hyvönen, M., & Kemp, P. R. (2003). Adhesion of Endothelial Cells to NOV Is Mediated by the Integrins αvβ3 and α5β1. *Journal of Vascular Research*, *40*(3), 234–243. https://doi.org/10.1159/000071887

Epenetos, A. A., Snook, D., Durbin, H., Johnson, P. M., & Taylor-Papadimitriou, J. (1986). Limitations of Radiolabeled Monoclonal Antibodies for Localization of Human Neoplasms. *Cancer Research*, *46*(6), 3183–3191.

Feng, D., & Gerarduzzi, C. (2020). Emerging Roles of Matricellular Proteins in Systemic Sclerosis. *International Journal of Molecular Sciences*, *21*, 4776. https://doi.org/10.3390/ijms21134776

Feng, T., Meng, J., Kou, S., Jiang, Z., Huang, X., Lu, Z., Zhao, H., Lau, L. F., Zhou, B., & Zhang, H. (2019). CCN1-Induced Cellular Senescence Promotes Heart Regeneration. *Circulation*, *139*(21), 2495–2498. https://doi.org/10.1161/CIRCULATIONAHA.119.039530

Funderburgh, J. L., Mann, M. M., & Funderburgh, M. L. (2003). Keratocyte Phenotype Mediates Proteoglycan Structure: A Role For Fibroblasts in Corneal Fibrosis. *Journal of Biological Chemistry*, *278*(46), 45629–45637. https://doi.org/10.1074/JBC.M303292200

Gabbiani, G. (2003). The myofibroblast in wound healing and fibrocontractive diseases. In *Journal of Pathology*. https://doi.org/10.1002/path.1427

Gabbiani, G., Ryan, G., & Majne, G. (1971). Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia*, *27*(5), 549–550. https://doi.org/10.1007/BF02147594

Gabrielli, A., Avvedimento, E. v., & Krieg, T. (2009). Scleroderma. *New England Journal of Medicine*, *360*(19), 1989–2003. https://doi.org/10.1056/NEJMRA0806188

Gao, R., & Brigstock, D. R. (2004). Connective Tissue Growth Factor (CCN2) Induces Adhesion of Rat Activated Hepatic Stellate Cells by Binding of Its C-terminal Domain to Integrin αvβ3 and Heparan Sulfate Proteoglycan *. *Journal of Biological Chemistry*, *279*(10), 8848–8855. https://doi.org/10.1074/JBC.M313204200

Gardner, H., Shearstone, J. R., Bandaru, R., Crowell, T., Lynes, M., Trojanowska, M., Pannu, J., Smith, E., Jablonska, S., Blaszczyk, M., Tan, F. K., & Mayes, M. D. (2006). Gene profiling of scleroderma skin reveals robust signatures of disease that are imperfectly reflected in the transcript profiles of explanted fibroblasts. *Arthritis & Rheumatology*, *54*(6), 1961–1973. https://doi.org/10.1002/ART.21894

George, J., & Tsutsumi, M. (2007). siRNA-mediated knockdown of connective tissue growth factor prevents N-nitrosodimethylamine-induced hepatic fibrosis in rats. *Gene Therapy*, *14*(10), 790–803. https://doi.org/10.1038/sj.gt.3302929

Graness, A., Cicha, I., & Goppelt-Struebe, M. (2006). Contribution of Src-FAK signaling to the induction of connective tissue growth factor in renal fibroblasts. *Kidney International*, *69*(8), 1341–1349. https://doi.org/10.1038/SJ.KI.5000296

Grazioli, S., Gil, S., An, D., Kajikawa, O., Farnand, A. W., Hanson, J. F., Birkland, T., Chen, P., Duffield, J., Schnapp, L. M., Altemeier, W. A., & Matute-Bello, G. (2015). CYR61 (CCN1) overexpression induces lung injury in mice. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *308*(8), L765. https://doi.org/10.1152/AJPLUNG.00190.2014

Gressner, A. M., Yagmur, E., Lahme, B., Gressner, O., & Stanzel, S. (2006). Connective Tissue Growth Factor in Serum as a New Candidate Test for Assessment of Hepatic Fibrosis. *Clinical Chemistry*, *52*(9), 1815–1817. https://doi.org/10.1373/CLINCHEM.2006.070466

Grotendorst, G. R., & Duncan, M. R. (2005). Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. *FASEB Journal*, *19*(7), 729–738. https://doi.org/10.1096/FJ.04-3217COM

Györfi, A. H., Matei, A. E., & Distler, J. H. W. (2018). Targeting TGF-β signaling for the treatment of fibrosis. *Matrix Biology*, *68–69*, 8–27. https://doi.org/10.1016/J.MATBIO.2017.12.016

Harding, F. A., Stickler, M. M., Razo, J., & DuBridge, R. (2010). The immunogenicity of humanized and fully human antibodies. *MAbs*, *2*(3), 256–265. https://doi.org/10.4161/MABS.2.3.11641

Hinz, B. (2010). The myofibroblast: Paradigm for a mechanically active cell. *Journal of Biomechanics*. https://doi.org/10.1016/j.jbiomech.2009.09.020

Hinz, B. (2016). The role of myofibroblasts in wound healing. *Current Research in Translational Medicine*, *64*(4), 171–177. https://doi.org/10.1016/J.RETRAM.2016.09.003

Hinz, B., Celetta, G., Tomasek, J. J., Gabbiani, G., & Chaponnier, C. (2017). Alpha-Smooth Muscle Actin Expression Upregulates Fibroblast Contractile Activity. *Molecular Biology of the Cell*, *12*(9), 2730–2741. https://doi.org/10.1091/MBC.12.9.2730

Hinz, B., McCulloch, C. A., & Coelho, N. M. (2019). Mechanical regulation of myofibroblast phenoconversion and collagen contraction. *Experimental Cell Research*, *379*(1), 119–128. https://doi.org/10.1016/J.YEXCR.2019.03.027

Holbourn, K. P., Acharya, K. R., & Perbal, B. (2008). The CCN family of proteins: structure–function relationships. *Trends in Biochemical Sciences*, *33*(10), 473. https://doi.org/10.1016/J.TIBS.2008.07.006

Holmes, A., Abraham, D. J., Chen, Y., Denton, C., Shi-Wen, X., Black, C. M., & Leask, A. (2003). Constitutive Connective Tissue Growth Factor Expression in Scleroderma Fibroblasts Is Dependent on Sp1. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M305019200

Holmes, A., Abraham, D. J., Sa, S., Shiwen, X., Black, C. M., & Leask, A. (2001). CTGF and SMADs, Maintenance of Scleroderma Phenotype Is Independent of SMAD Signaling. *Journal of Biological Chemistry*, *276*(14), 10594–10601. https://doi.org/10.1074/jbc.M010149200

Hong, L., Lai, H.-L., Fang, Y., Tao, Y., & Qiu, Y. (2018). Silencing CTGF/CCN2 inactivates the MAPK signaling pathway to alleviate myocardial fibrosis and left ventricular hypertrophy in rats with dilated cardiomyopathy. *Journal of Cellular Biochemistry*, *119*(11), 9519–9531. https://doi.org/10.1002/JCB.27268

Hoshijima, M., Hattori, T., Inoue, M., Araki, D., Hanagata, H., Miyauchi, A., & Takigawa, M. (2006). CT domain of CCN2/CTGF directly interacts with fibronectin and enhances cell adhesion of chondrocytes through integrin α5β1. *FEBS Letters*, *580*(5), 1376–1382. https://doi.org/10.1016/J.FEBSLET.2006.01.061

Humphreys, B. D., Lin, S.-L., Kobayashi, A., Hudson, T. E., Nowlin, B. T., Bonventre, J. v, Todd Valerius, M., McMahon, A. P., & Duffield, J. S. (2010). Epithelial and Mesenchymal Cell Biology Fate Tracing Reveals the Pericyte and Not Epithelial Origin of Myofibroblasts in Kidney Fibrosis. *American Journal of Pathology*, *176*, 85–97. https://doi.org/10.2353/ajpath.2010.090517

Hung, C., Linn, G., Chow, Y.-H., Kobayashi, A., Mittelsteadt, K., Altemeier, W. A., Gharib, S. A., Schnapp, L. M., & Duffield, J. S. (2013). Role of Lung Pericytes and Resident Fibroblasts in the Pathogenesis of Pulmonary Fibrosis. *American Journal*

of Respiratory and Critical Care Medicine, *188*(7), 820–830. https://doi.org/10.1164/RCCM.201212-2297OC

Hutchenreuther, J., & Leask, A. (2016). A tale of two orgins: do myofibroblasts originate from different sources in wound healing and fibrosis? *Cell and Tissue Research*, *365*(3), 507–509. https://doi.org/10.1007/S00441-016-2419-5

Igarashi, A., Nashiro, K., Kikuchi, K., Sato, S., Ihn, H., Fujimoto, M., Grotendorst, G. R., & Takehara, K. (1996). Connective Tissue Growth Factor Gene Expression in Tissue Sections From Localized Scleroderma, Keloid, and Other Fibrotic Skin Disorders. *Journal of Investigative Dermatology*, *106*(4), 729–733. https://doi.org/10.1111/1523-1747.ep12345771

Ishida, W., Mori, Y., Lakos, G., Sun, L., Shan, F., Bowes, S., Josiah, S., Lee, W. C., Singh, J., Ling, L. E., & Varga, J. (2006). Intracellular TGF-β receptor blockade abrogates smad-dependent fibroblast activation in vitro and in vivo. *Journal of Investigative Dermatology*. https://doi.org/10.1038/sj.jid.5700303

Iwaisako, K., Jiang, C., Zhang, M., Cong, M., Moore-Morris, T. J., Park, T. J., Liu, X., Xu, J., Wang, P., Paik, Y.-H., Meng, F., Asagiri, M., Murray, L. A., Hofmann, A. F., Iida, T., Glass, C. K., Brenner, D. A., & Kisseleva, T. (2014). Origin of myofibroblasts in the fibrotic liver in mice. *Proceedings of the National Academy of Sciences*, *111*(32), E3297–E3305. https://doi.org/10.1073/PNAS.1400062111

Jia, Q., Xue, T., Zhang, Q., Cheng, W., Zhang, C., Ma, J., Bu, Y., Yu, S., & Liu, Q. (2017). CCN3 is a therapeutic target relating enhanced stemness and coagulation in hepatocellular carcinoma. *Scientific Reports*, *7*(1), 13846. https://doi.org/10.1038/S41598-017-14087-4

Joe, A. W. B., Yi, L., Natarajan, A., Grand, F. le, So, L., Wang, J., Rudnicki, M. A., & Rossi, F. M. v. (2010). Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. *Nature Cell Biology*, *12*(2), 163. https://doi.org/10.1038/NCB2015

John, A. E., Graves, R. H., Pun, K. T., Vitulli, G., Forty, E. J., Mercer, P. F., Morrell, J. L., Barrett, J. W., Rogers, R. F., Hafeji, M., Bibby, L. I., Gower, E., Morrison, V. S., Man, Y., Roper, J. A., Luckett, J. C., Borthwick, L. A., Barksby, B. S., Burgoyne, R. A., … Slack, R. J. (2020). Translational pharmacology of an inhaled small molecule αvβ6 integrin inhibitor for idiopathic pulmonary fibrosis. *Nature Communications*, *11*(1), 4659. https://doi.org/10.1038/S41467-020-18397-6

Joliot, V., Martinerie, C., Dambrine, G., Plassiart, G., Brisac, M., Crochet, J., & Perbal, B. (1992). Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. *Molecular and Cellular Biology*, *12*(1), 21. https://doi.org/10.1128/MCB.12.1.10

Jun, J.-I., & Lau, L. F. (2011). Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. *Nature Reviews Drug Discovery*, *10*(12), 945–963. https://doi.org/10.1038/nrd3599

Jun, J.-I., & Lau, L. F. (2017). CCN2 induces cellular senescence in fibroblasts. *Journal of Cell Communication and Signaling*, *11*(1), 23. https://doi.org/10.1007/S12079-016-0359-1

Kane, C. J. M., Hebda, P. A., Mansbridge, J. N., & Hanawalt, P. C. (1991). Direct evidence for spatial and temporal regulation of transforming growth factor β1 expression during cutaneous wound healing. *Journal of Cellular Physiology*, *148*(1), 157–173. https://doi.org/10.1002/JCP.1041480119

Kanisicak, O., Khalil, H., Ivey, M. J., Karch, J., Maliken, B. D., Correll, R. N., Brody, M. J., Lin, S.-C. J., Aronow, B. J., Tallquist, M. D., & Molkentin, J. D. (2016). Genetic lineage tracing defines myofibroblast origin and function in the injured heart. *Nature Communications*, *7*, 12260. https://doi.org/10.1038/NCOMMS12260

Kawaki, H., Kubota, S., Suzuki, A., Lazar, N., Yamada, T., Matsumura, T., Ohgawara, T., Maeda, T., Perbal, B., Lyons, K. M., & Takigawa, M. (2008). Cooperative Regulation of Chondrocyte Differentiation by CCN2 and CCN3 Shown by a Comprehensive Analysis of the CCN Family Proteins in Cartilage. *Journal of Bone and Mineral Research*, *23*, 1751–1764. https://doi.org/10.1359/JBMR.080615

Kendall, R. T., & Feghali-Bostwick, C. A. (2014). Fibroblasts in fibrosis: Novel roles and mediators. In *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2014.00123

Kielty, C. M., & Shuttleworth, C. A. (1997). Microfibrillar elements of the dermal matrix. *Microscopy Research and Technique*, *38*(4), 413–427. https://doi.org/10.1002/(SICI)1097-0029(19970815)38:4<413::AID-JEMT9>3.0.CO;2-J

Kim, K.-H., Chen, C.-C., Monzon, R. I., & Lau, L. F. (2013). Matricellular Protein CCN1 Promotes Regression of Liver Fibrosis through Induction of Cellular Senescence in Hepatic Myofibroblasts. *Molecular and Cellular Biology*, *33*(10). https://doi.org/10.1128/MCB.00049-13

King, J., Abraham, D., & Stratton, R. (2018). Chemokines in systemic sclerosis. *Immunology Letters*, *195*, 68–75. https://doi.org/10.1016/J.IMLET.2017.12.001

Kinoshita, K., Aono, Y., Azuma, M., Kishi, J., Takezaki, A., Kishi, M., Makino, H., Okazaki, H., Uehara, H., Izumi, K., Sone, S., & Nishioka, Y. (2013). Antifibrotic Effects of Focal Adhesion Kinase Inhibitor in Bleomycin-Induced Pulmonary Fibrosis in Mice. *American Journal of Respiratory Cell and Molecular Biology*, *49*(4), 536–543. https://doi.org/10.1165/RCMB.2012-0277OC

Kireeva, M. L., MO, F. E., Yang, G. P., & Lau, L. F. (1996). Cyr61, a product of a growth factor-inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. *Molecular and Cellular Biology*, *16*(4), 1334. https://doi.org/10.1128/MCB.16.4.1326

Kissin, E. Y., Lemaire, R., Korn, J. H., & Lafyatis, R. (2002). Transforming growth factor β induces fibroblast fibrillin-1 matrix formation. *Arthritis & Rheumatology*, *46*(11), 3000–3009. https://doi.org/10.1002/ART.10621

Kissin, E. Y., Merkel, P. A., & Lafyatis, R. (2006). Myofibroblasts and hyalinized collagen as markers of skin disease in systemic sclerosis. *Arthritis & Rheumatology*, *54*(11), 3655–3660. https://doi.org/10.1002/ART.22186

Koitabashi, N., Arai, M., Niwano, K., Watanabe, A., Endoh, M., Suguta, M., Yokoyama, T., Tada, H., Toyama, T., Adachi, H., Naito, S., Oshima, S., Nishida, T., Kubota, S., Takigawa, M., & Kurabayashi, M. (2008). Plasma connective tissue growth factor is a novel potential biomarker of cardiac dysfunction in patients with chronic heart failure. *European Journal of Heart Failure*, *10*(4), 373–379. https://doi.org/10.1016/J.EJHEART.2008.02.011

Kopp, J. L., Ormsbee, B. D., Desler, M., & Rizzino, A. (2008). Small Increases in the Level of Sox2 Trigger the Differentiation of Mouse Embryonic Stem Cells. *Stem Cells*, *26*(4), 903–911. https://doi.org/10.1634/STEMCELLS.2007-0951

Kreuter, A., Höxtermann, S., Tigges, C., Hahn, S. A., Altmeyer, P., & Gambichler, T. (2009). Clonal T-cell populations are frequent in the skin and blood of patients with systemic sclerosis. *British Journal of Dermatology*, *161*(4), 785–790. https://doi.org/10.1111/J.1365-2133.2009.09213.X

Krupska, I., Bruford, E. A., & Chaqour, B. (2015). Eyeing the Cyr61/CTGF/NOV (CCN) group of genes in development and diseases: highlights of their structural likenesses and functional dissimilarities. *Human Genomics*, *9*(24), 1–13. https://doi.org/10.1186/S40246-015-0046-Y

Kuk, H., Hutchenreuther, J., Murphy-Marshman, H., Carter, D., & Leask, A. (2015). 5Z-7-Oxozeanol Inhibits the Effects of TGFβ1 on Human Gingival Fibroblasts. *PLoS ONE*, *10*(4), e0123689. https://doi.org/10.1371/JOURNAL.PONE.0123689

Lacouture, M. E., Morris, J. C., Lawrence, D. P., Tan, A. R., Thomas, ·, Olencki, E., Shapiro, G. I., Dezube, B. J., Berzofsky, J. A., Hsu, F. J., Guitart, J., Lacouture, M. E., Morris, J. C., Berzofsky, J. A., Lawrence, D. P., Tan, A. R., Olencki, T. E., Shapiro, G. I., Dezube, B. J., … Guitart, J. (2015). Cutaneous keratoacanthomas/squamous cell carcinomas associated with neutralization of transforming growth factor β by the monoclonal antibody fresolimumab (GC1008). *Cancer Immunology, Immunotherapy*, *3*, 437–446. https://doi.org/10.1007/s00262-015-1653-0

Lafont, J., Thibout, H., Dubois, C., Laurent, M., & Martinerie, C. (2009). NOV/CCN3 Induces Adhesion of Muscle Skeletal Cells and Cooperates with FGF2 and IGF-1 to Promote Proliferation and Survival. *Cell Communication & Adhesion*, *12*(1–2), 41–57. https://doi.org/10.1080/15419060500383069

Lagares, D., Busnadiego, O., Ana García-Fernández, R., Kapoor, M., Liu, S., Carter, D. E., Abraham, D., Shi-Wen, X., Carreira, P., Fontaine, B. A., Shea, B. S., Tager, A. M., Leask, A., Lamas, S., & Rodríguez-Pascual, F. (2012). Inhibition of focal adhesion kinase prevents experimental lung fibrosis and myofibroblast formation. *Arthritis & Rheumatology*, *64*(5), 1653–1664. https://doi.org/10.1002/art.33482

Lai, C.-F., Chen, Y.-M., Chiang, W.-C., Lin, S.-L., Kuo, M.-L., & Tsai, T.-J. (2013). Cysteine-Rich Protein 61 Plays a Proinflammatory Role in Obstructive Kidney

Fibrosis. *PLoS ONE*, *8*(2), 56481. https://doi.org/10.1371/JOURNAL.PONE.0056481

Lai, C.-F., Lin, S.-L., Chiang, W.-C., Chen, Y.-M., Wu, V.-C., Young, G.-H., Ko, W.-J., Kuo, M.-L., Tsai, T.-J., & Wu, K.-D. (2014). Blockade of cysteine-rich protein 61 attenuates renal inflammation and fibrosis after ischemic kidney injury. *American Journal of Physiology - Renal Physiology*, *307*(5), 581–592. https://doi.org/10.1152/AJPRENAL.00670.2013

Latinkic, B. v., Mercurio, S., Bennett, B., Hirst, E. M. A., Xu, Q., Lau, L. F., Mohun, T. J., & Smith, J. C. (2003). Xenopus Cyr61 regulates gastrulation movements and modulates Wnt signalling. *Development*, *130*(11), 2429–2441. https://doi.org/10.1242/DEV.00449

Lau, L. F. (2011). CCN1/CYR61: the very model of a modern matricellular protein. *Cellular and Molecular Life Sciences*, *68*(19). https://doi.org/10.1007/s00018-011-0778-3

Lau, L. F. (2016). Cell surface receptors for CCN proteins. *Journal of Cell Communication and Signaling*, *10*(2), 127. https://doi.org/10.1007/S12079-016-0324-Z

Leask, A. (2015). Matrix remodeling in systemic sclerosis. *Seminars in Immunopathology*. https://doi.org/10.1007/s00281-015-0508-2

Leask, A. (2020a). The hard problem: Mechanotransduction perpetuates the myofibroblast phenotype in scleroderma fibrosis. *Wound Repair and Regeneration*, *29*, 582–587. https://onlinelibrary-wiley-com.proxy1.lib.uwo.ca/doi/pdfdirect/10.1111/wrr.12889

Leask, A. (2020b). Conjunction junction, what's the function? CCN proteins as targets in fibrosis and cancers. *American Journal of Physiology - Cell Physiology*, *318*(6), C1046. https://doi.org/10.1152/AJPCELL.00028.2020

Leask, A., & Abraham, D. J. (2006). All in the CCN family: essential matricellular signaling modulators emerge from the bunker. *Journal of Cell Science*. https://doi.org/10.1242/jcs.03270

Leask, A., Holmes, A., Black, C. M., & Abraham, D. J. (2003). Connective tissue growth factor gene regulation: Requirements for its induction by transforming growth factor-β2 in fibroblasts. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M210366200

Lemaire, R., Farina, G., Bayle, J., Dimarzio, M., Pendergrass, S. A., Milano, A., Whitfield, M. L., & Lafyatis, R. (2010). Antagonistic Effect of the Matricellular Signaling Protein CCN3 on TGF-β- and Wnt-Mediated Fibrillinogenesis in Systemic Sclerosis and Marfan Syndrome. *Journal of Investigative Dermatology*, *130*(6), 1514–1523. https://doi.org/10.1038/JID.2010.15

Lin, C. G., Chen, C.-C., Leu, S.-J., Grzeszkiewicz, T. M., & Lau, L. F. (2005). Integrin-dependent Functions of the Angiogenic Inducer NOV (CCN3): IMPLICATION IN WOUND HEALING *. *Journal of Biological Chemistry*, *280*(9), 8229–8237. https://doi.org/10.1074/JBC.M404903200

Lin, S.-L., Kisseleva, T., Brenner, D. A., & Duffield, J. S. (2008). Pericytes and Perivascular Fibroblasts Are the Primary Source of Collagen-Producing Cells in Obstructive Fibrosis of the Kidney. *American Journal of Pathology*, *173*(6), 1617–1627. https://doi.org/10.2353/AJPATH.2008.080433

Liu, S., Herault, Y., Pavlovic, G., & Leask, A. (2014). Skin progenitor cells contribute to bleomycin-induced skin fibrosis. *Arthritis & Rheumatology*. https://doi.org/10.1002/art.38276

Liu, S., Kapoor, M., Denton, C. P., Abraham, D. J., & Leask, A. (2009). Loss of Beta1 Integrin in Mouse Fibroblasts Results in Resistance to Skin Scleroderma in a Mouse Model. *Arthritis & Rheumatology*, *60*(9), 2817–2821. https://doi.org/10.1002/art.24801

Liu, S., Parapuram, S. K., & Leask, A. (2013). Fibrosis Caused by Loss of PTEN Expression in Mouse Fibroblasts Is Crucially Dependent on CCN2. *Arthritis & Rheumatology*, *65*(11), 2940–2944. https://doi.org/10.1002/ART.38121

Liu, S., Shi-Wen, X., Abraham, D. J., & Leask, A. (2011). CCN2 is required for bleomycin-induced skin fibrosis in mice. *Arthritis & Rheumatology*, *63*(1), 239–246. https://doi.org/10.1002/art.30074

Liu, S., Shi-wen, X., Kennedy, L., Pala, D., Chen, Y., Eastwood, M., Carter, D. E., Black, C. M., Abraham, D. J., & Leask, A. (2007). FAK Is Required for TGFβ-induced JNK Phosphorylation in Fibroblasts: Implications for Acquisition of a Matrix-remodeling Phenotype. *Molecular Biology of the Cell*. https://doi.org/10.1091/mbc.E06-12-1121

Liu, S., Shi-wen, X., Blumbach, K., Eastwood, M., Denton, C. P., Eckes, B., Krieg, T., Abraham, D. J., & Leask, A. (2010). Expression of integrin β1 by fibroblasts is required for tissue repair in vivo. *Journal of Cell Science*, *123*(21), 3674–3682. https://doi.org/10.1242/JCS.070672

Liu, S., Thompson, K., & Leask, A. (2014). CCN2 expression by fibroblasts is not required for cutaneous tissue repair. *Wound Repair and Regeneration*, *22*(1), 119–124. https://doi.org/10.1111/wrr.12131

llić, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., & Aizawa, S. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, *377*(6549), 539–544. https://doi.org/10.1038/377539a0

Makino, K., Makino, T., Stawski, L., Lipson, K. E., Leask, A., & Trojanowska, M. (2017). Anti-connective tissue growth factor (CTGF/CCN2) monoclonal antibody attenuates skin fibrosis in mice models of systemic sclerosis. *Arthritis Research & Therapy*, *19*(1), 134. https://doi.org/10.1186/s13075-017-1356-3

Malmstrom, J., Linberg, H., Lindberg, C., Bratt, C., Wieslander, E., Delander, E. L., Särnstrand, B., Burns, J. S., Mose-Larsen, P., Fey, S., & Marko-Varga, G. (2004). Transforming Growth Factor-β1 Specifically Induce Proteins Involved in the Myofibroblast Contractile Apparatus. *Molecular & Cellular Proteomics*, *3*(5), 466–477. https://doi.org/10.1074/MCP.M300108-MCP200

Manetti, M., Guiducci, S., Romano, E., Avouac, J., Rosa, I., Ruiz, B., Lepri, G., Bellando-Randone, S., Ibba-Manneschi, L., Allanore, Y., & Matucci-Cerinic, M. (2013). Decreased expression of the endothelial cell-derived factor EGFL7 in systemic sclerosis: potential contribution to impaired angiogenesis and vasculogenesis. *Arthritis Research & Therapy*, *15*(5), R165. https://doi.org/10.1186/AR4349

Marino, G. K., Santhiago, M. R., Santhanam, A., Torricelli, A. A. M., & Wilson, S. E. (2017). Regeneration of defective epithelial basement membrane and restoration of corneal transparency after photorefractive keratectomy. *Journal of Refractive Surgery*, *33*(5), 337–346. https://doi.org/10.3928/1081597X-20170126-02

Massagué, J. (1998). TGFβ Signal Transduction. *Annual Review of Biochemistry*, *67*, 753–791. https://doi.org/10.1146/ANNUREV.BIOCHEM.67.1.753

Maurer, B., Distler, A., Suliman, Y. A., Gay, R. E., Michel, B. A., Gay, S., Distler, J. H. W., & Distler, O. (2014). Vascular endothelial growth factor aggravates fibrosis and vasculopathy in experimental models of systemic sclerosis. *Annals of the Rheumatic Diseases*, *73*(10), 1880–1887. https://doi.org/10.1136/ANNRHEUMDIS-2013-203535

McCray, C. J., & Mayes, M. D. (2015). Update on systemic sclerosis. *Current Allergy and Asthma Reports*. https://doi.org/10.1007/s11882-015-0526-0

Mederacke, I., Hsu, C. C., Troeger, J. S., Huebener, P., Mu, X., Dapito, D. H., Pradere, J.-P., & Schwabe, R. F. (2013). Fate-tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its etiology. *Nature Communications*, *4*, 2823. https://doi.org/10.1038/NCOMMS3823

Mimura, Y., Ihn, H., Jinnin, M., Asano, Y., Yamane, K., & Tamaki, K. (2005). Constitutive Phosphorylation of Focal Adhesion Kinase Is Involved in the Myofibroblast Differentiation of Scleroderma Fibroblasts. *Journal of Investigative Dermatology*, *124*(5), 886–892. https://doi.org/10.1111/J.0022-202X.2005.23701.X

Mori, T., Kawara, S., Shinozaki, M., Hayashi, N., Kakinuma, T., Igarashi, A., Takigawa, M., Nakanishi, T., & Takehara, K. (1999). Role and Interaction of Connective Tissue Growth Factor With Transforming Growth Factor-β in Persistent Fibrosis: A Mouse Fibrosis Model. *Journal of Cellular Physiology*, *181*, 153–159.

Morris, J. C., Tan, A. R., Olencki, T. E., Shapiro, G. I., Dezube, B. J., Reiss, M., Hsu, F. J., Berzofsky, J. A., & Lawrence, D. P. (2014). Phase I Study of GC1008 (Fresolimumab): A Human Anti-Transforming Growth Factor-Beta (TGFβ) Monoclonal Antibody in Patients with Advanced Malignant Melanoma or Renal Cell Carcinoma. *PLoS ONE*, *9*(3), e90353. https://doi.org/10.1371/JOURNAL.PONE.0090353

Murphy-Marshman, H., Quensel, K., Xu, S. W., Barnfield, R., Kelly, J., Peidl, A., Stratton, R. J., & Leask, A. (2017). Antioxidants and NOX1/NOX4 inhibition blocks TGFβ1-induced CCN2 and α-SMA expression in dermal and gingival fibroblasts. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0186740

Nallet-Staub, F., Marsaud, V., Li, L., Gilbert, C., Dodier, S., Bataille, V., Sudol, M., Herlyn, M., & Mauviel, A. (2014). Pro-Invasive Activity of the Hippo Pathway

Effectors YAP and TAZ in Cutaneous Melanoma. *Journal of Investigative Dermatology*, *134*(1), 123–132. https://doi.org/10.1038/JID.2013.319

Nguyen, T. Q., Tarnow, L., Andersen, S., Hovind, P., Parving, H.-H., Goldschmeding, R., & Nieuwenhoven, F. A. van. (2006). Urinary Connective Tissue Growth Factor Excretion Correlates With Clinical Markers of Renal Disease in a Large Population of Type 1 Diabetic Patients With Diabetic Nephropathy. *Diabetes Care*, *29*(1), 83–88. https://doi.org/10.2337/DIACARE.29.01.06.DC05-1670

Nishida, T., Kubota, S., Fukunaga, T., Kondo, S., Yosimichi, G., Nakanishi, T., Takano-Yamamoto, T., & Takigawa, M. (2003). CTGF/Hcs24, hypertrophic chondrocyte-specific gene product, interacts with perlecan in regulating the proliferation and differentiation of chondrocytes. *Journal of Cellular Physiology*, *196*(2), 265–275. https://doi.org/10.1002/JCP.10277

O'Brien, T. P., Yang, G. P., Sanders, L., & Lau, L. F. (1990). Expression of cyr61, a growth factor-inducible immediate-early gene. *Molecular and Cellular Biology*, *10*(7), 3577. https://doi.org/10.1128/MCB.10.7.3569

Pang, X.-J., Liu, X.-J., Liu, Y., Liu, W.-B., Li, Y.-R., Yu, G.-X., Tian, X.-Y., Zhang, Y.-B., Song, J., Jin, C.-Y., & Zhang, S.-Y. (2021). Drug Discovery Targeting Focal Adhesion Kinase (FAK) as a Promising Cancer Therapy. *Molecules*, *26*(14), 4250. https://doi.org/10.3390/MOLECULES26144250

Parapuram, S. K., Shi-Wen, X., Elliott, C., Welch, I. D., Jones, H., Baron, M., Denton, C. P., Abraham, D. J., & Leask, A. (2011). Loss of PTEN expression by dermal fibroblasts causes skin fibrosis. *Journal of Investigative Dermatology*. https://doi.org/10.1038/jid.2011.156

Parapuram, S. K., Thompson, K., Tsang, M., Hutchenreuther, J., Bekking, C., Liu, S., & Leask, A. (2015). Loss of PTEN expression by mouse fibroblasts results in lung fibrosis through a CCN2-dependent mechanism. *Matrix Biology*. https://doi.org/10.1016/j.matbio.2015.01.017

Parker, M. W., Rossi, D., Peterson, M., Smith, K., Sikström, K., White, E. S., Connett, J. E., Henke, C. A., Larsson, O., & Bitterman, P. B. (2014). Fibrotic extracellular matrix activates a profibrotic positive feedback loop. *Journal of Clinical Investigation*, *124*(4), 1635. https://doi.org/10.1172/JCI71386

Perbal, B. (2001). NOV (nephroblastoma overexpressed) and the CCN family of genes: Structural and functional issues. In *Journal of Clinical Pathology - Molecular Pathology*. https://doi.org/10.1136/mp.54.2.57

Perbal, B., Tweedie, S., & Bruford, E. (2018). The official unified nomenclature adopted by the HGNC calls for the use of the acronyms, CCN1–6, and discontinuation in the use of CYR61, CTGF, NOV and WISP 1–3 respectively. *Journal of Cell Communication and Signaling*, *12*(4), 625–629. https://doi.org/10.1007/s12079-018-0491-1

Petrosino, J. M., Leask, A., & Accornero, F. (2019). Genetic manipulation of CCN2/CTGF unveils cell-specific ECM-remodeling effects in injured skeletal muscle. *FASEB Journal*, *33*(2), 2047–2057. https://doi.org/10.1096/fj.201800622RR

Phanish, M. K., Winn, S. K., & Dockrell, M. E. C. (2010). Connective Tissue Growth Factor-(CTGF, CCN2) – A Marker, Mediator and Therapeutic Target for Renal Fibrosis. *Nephron Experimental Nephrology*, *114*(3), e83–e92. https://doi.org/10.1159/000262316

Pi, L., Robinson, P. M., Jorgensen, M., Oh, S.-H., Brown, A. R., Weinreb, P. H., Trinh, T. le, Yianni, P., Liu, C., Leask, A., Violette, S. M., Scott, E. W., Schultz, G. S., & Petersen, B. E. (2015). Connective Tissue Growth Factor and Integrin αvβ6: a New Pair of Regulators Critical for Ductular Reaction and Biliary Fibrosis. *Hepatology*, *61*(2), 691. https://doi.org/10.1002/HEP.27425

Piersma, B., Hayward, M. K., & Weaver, V. M. (2020). Fibrosis and cancer: A strained relationship. *Biochimica et Biophysica Acta. Reviews On Cancer*, *1873*(2), 188356. https://doi.org/10.1016/J.BBCAN.2020.188356

Ponticos, M., Holmes, A. M., Shi-Wen, X., Leoni, P., Khan, K., Rajkumar, V. S., Hoyles, R. K., Bou-Gharios, G., Black, C. M., Denton, C. P., Abraham, D. J., Leask, A., & Lindahl, G. E. (2009). Pivotal role of connective tissue growth factor in lung fibrosis: MAPK-dependent transcriptional activation of type I collagen. *Arthritis & Rheumatology*, *60*(7), 2142–2155. https://doi.org/10.1002/ART.24620

Prakoura, N., & Chatziantoniou, C. (2017). Matricellular Proteins and Organ Fibrosis. *Current Patho-Biology Reports*, 1–11. https://doi.org/10.1007/s40139-017-0138-6ï

Quesnel, K., Shi-wen, X., Hutchenreuther, J., Xiao, Y., Liu, S., Peidl, A., Naskar, D., Siqueira, W. L., O'Gorman, D. B., Hinz, B., Stratton, R. J., & Leask, A. (2019). CCN1 expression by fibroblasts is required for bleomycin-induced skin fibrosis. *Matrix Biology Plus*, *3*, 100009. https://doi.org/10.1016/J.MBPLUS.2019.100009

Raghu, G., Scholand, M. B., de Andrade, J., Lancaster, L., Mageto, Y., Goldin, J., Brown, K. K., Flaherty, K. R., Wencel, M., Wanger, J., Neff, T., Valone, F., Stauffer, J., & Porter, S. (2016). FG-3019 anti-connective tissue growth factor monoclonal antibody: Results of an open-label clinical trial in idiopathic pulmonary fibrosis. *European Respiratory Journal*, *47*, 1481–1491. https://doi.org/10.1183/13993003.01030-2015

Ren, Z., Hou, Y., Ma, S., Tao, Y., Li, J., Cao, H., & Ji, L. (2014). Effects of CCN3 on fibroblast proliferation, apoptosis and extracellular matrix production. *International Journal of Molecular Medicine*, *33*(6), 1607–1612. https://doi.org/10.3892/IJMM.2014.1735

Resovi, A., Borsotti, P., Ceruti, T., Passoni, A., Zucchetti, M., Berndt, A., Riser, B. L., Taraboletti, G., & Belotti, D. (2020). CCN-Based Therapeutic Peptides Modify Pancreatic Ductal Adenocarcinoma Microenvironment and Decrease Tumor Growth in Combination with Chemotherapy. *Cells*, *9*(4), 952. https://doi.org/10.3390/CELLS9040952

Rice, L. M., Padilla, C. M., McLaughlin, S. R., Mathes, A., Ziemek, J., Goummih, S., Nakerakanti, S., York, M., Farina, G., Whitfield, M. L., Spiera, R. F., Christmann, R. B., Gordon, J. K., Weinberg, J., Simms, R. W., & Lafyatis, R. (2015). Fresolimumab treatment decreases biomarkers and improves clinical symptoms in

systemic sclerosis patients. *Journal of Clinical Investigation*, *125*(7), 2807. https://doi.org/10.1172/JCI77958

Rinkevich, Y., Walmsley, G. G., Hu, M. S., Maan, Z. N., Newman, A. M., Drukker, M., Januszyk, M., Krampitz, G. W., Gurtner, G. C., Lorenz, H. P., Weissman, I. L., & Longaker, M. T. (2015). Identification and isolation of a dermal lineage with intrinsic fibrogenic potential. *Science*, *348*(6232), aaa2151. https://doi.org/10.1126/SCIENCE.AAA2151

Riser, B. L., Barnes, J. L., & Varani, J. (2015). Balanced regulation of the CCN family of matricellular proteins: a novel approach to the prevention and treatment of fibrosis and cancer. *Journal of Cell Communication and Signaling*, *9*(4), 327. https://doi.org/10.1007/s12079-015-0309-3

Riser, B. L., Najmabadi, F., Garchow, K., Barnes, J. L., Peterson, D. R., & Sukowski, E. J. (2014). Treatment with the Matricellular Protein CCN3 Blocks and/or Reverses Fibrosis Development in Obesity with Diabetic Nephropathy. *American Journal of Pathology*, *184*(11), 2908–2921. https://doi.org/10.1016/j.ajpath.2014.07.009

Riser, B. L., Najmabadi, F., Perbal, B., Peterson, D. R., Rambow, J. A., Riser, M. L., Sukowski, E., Yeger, H., & Riser, S. C. (2009). CCN3 (NOV) is a negative regulator of CCN2 (CTGF) and a novel endogenous inhibitor of the fibrotic pathway in an in vitro model of renal disease. *American Journal of Pathology*, *174*(5), 1725–1734. https://doi.org/10.2353/ajpath.2009.080241

Riser, B. L., Najmabadi, F., Perbal, B., Rambow, J. A., Riser, M. L., Sukowski, E., Yeger, H., Riser, S. C., & Peterson, D. R. (2010). CCN3/CCN2 regulation and the fibrosis of diabetic renal disease. *Journal of Cell Communication and Signaling*, *4*(1), 39. https://doi.org/10.1007/s12079-010-0085-z

Rittié, L., Perbal, B., Castellot, J. J., Orringer, J. S., Voorhees, J. J., & Fisher, G. J. (2011). Spatial-temporal modulation of CCN proteins during wound healing in human skin in vivo. *Journal of Cell Communication and Signaling 2011 5:1*, *5*(1), 69–80. https://doi.org/10.1007/S12079-010-0114-Y

Roberts, A. B., Sporn, M. B., Assoian, R. K., Smith, J. M., Roche, N. S., Wakefield, L. M., Heine, U. I., Liotra, L. A., Falangat, V., Kehrl, J. H., & Faucit, A. S. (1986). Transforming growth factor type β: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, *83*, 4167–4171.

Roumm, A. D., Whiteside, T. L., Medsger, T. A., & Rodnan, G. P. (1984). Lymphocytes in the skin of patients with progressive systemic sclerosis. *Arthritis & Rheumatology*, *27*(6), 645–653. https://doi.org/10.1002/ART.1780270607

Sakai, N., Nakamura, M., Lipson, K. E., Miyake, T., Kamikawa, Y., Sagara, A., Shinozaki, Y., Kitajima, S., Toyama, T., Hara, A., Iwata, Y., Shimizu, M., Furuichi, K., Kaneko, S., Tager, A. M., & Wada, T. (2017). Inhibition of CTGF ameliorates peritoneal fibrosis through suppression of fibroblast and myofibroblast accumulation and angiogenesis. *Scientific Reports*, *7*(1), 5392. https://doi.org/10.1038/S41598-017-05624-2

Sakamoto, K., Yamaguchi, S., Ando, R., Miyawaki, A., Kabasawa, Y., Takagi, M., Li, C. L., Perbal, B., & Katsube, K. (2002). The Nephroblastoma Overexpressed Gene (NOV/ccn3) Protein Associates with Notch1 Extracellular Domain and Inhibits Myoblast Differentiation via Notch Signaling Pathway *. *Journal of Biological Chemistry*, *277*(33), 29399–29405. https://doi.org/10.1074/JBC.M203727200

Sargent, J. L., Milano, A., Bhattacharyya, S., Varga, J., Connolly, M. K., Chang, H. Y., & Whitfield, M. L. (2010). A TGFβ-Responsive Gene Signature Is Associated with a Subset of Diffuse Scleroderma with Increased Disease Severity. *Journal of Investigative Dermatology*, *130*(3), 694–705. https://doi.org/10.1038/JID.2009.318

Sato, S., Nagaoka, T., Hasegawa, M., Tamatani, T., Nakanishi, T., Takigawa, M., & Takehara, K. (2000). Serum levels of connective tissue growth factor are elevated in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. *Journal of Rheumatology*, *27*(1), 149–154. https://doi.org/10.1136/ARD.2005.040782

Sfikakis, P. P., McCune, B. K., Tsokos, M., Aroni, K., Vayiopoulos, G., & Tsokos, G. C. (1993). Immunohistological Demonstration of Transforming Growth Factor-β Isoforms in the Skin of Patients with Systemic Sclerosis. *Clinical Immunology and Immunopathology*, *69*(2), 199–204. https://doi.org/10.1006/CLIN.1993.1170

Schaller, M. D. (2010). Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *Journal of Cell Science*, *123*(7), 1007–1013. https://doi.org/10.1242/JCS.045112

Shimoyama, T., Hiraoka, S., Takemoto, M., Koshizaka, M., Tokuyama, H., Tokuyama, T., Watanabe, A., Fujimoto, M., Kawamura, H., Sato, S., Tsurutani, Y., Saito, Y., Perbal, B., Koseki, H., & Yokote, K. (2010). CCN3 Inhibits Neointimal Hyperplasia Through Modulation of Smooth Muscle Cell Growth and Migration. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *30*(4), 675–682. https://doi.org/10.1161/ATVBAHA.110.203356

Shi-wen, X., Liu, S., Eastwood, M., Sonnylal, S., Denton, C. P., Abraham, D. J., & Leask, A. (2009). Rac Inhibition Reverses the Phenotype of Fibrotic Fibroblasts. *PLoS ONE*, *4*(10). https://doi.org/10.1371/JOURNAL.PONE.0007438

Shi-wen, X., Racanelli, M., Ali, A., Simon, A., Quesnel, K., Stratton, R. J., & Leask, A. (2021). Verteporfin inhibits the persistent fibrotic phenotype of lesional scleroderma dermal fibroblasts. *Journal of Cell Communication and Signaling*, *15*(1), 71. https://doi.org/10.1007/S12079-020-00596-X

Shi-Wen, X., Thompson, K., Khan, K., Liu, S., Murphy-Marshman, H., Baron, M., Denton, C. P., Leask, A., & Abraham, D. J. (2012). Focal adhesion kinase and reactive oxygen species contribute to the persistent fibrotic phenotype of lesional scleroderma fibroblasts. *Rheumatology*. https://doi.org/10.1093/rheumatology/kes234

Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N., & Doetschman, T. (1992). Targeted disruption of the mouse transforming growth factor-β1 gene results in

multifocal inflammatory disease. *Nature*, *359*(6397), 693–699. https://doi.org/10.1038/359693a0

Singer, A. J., & Clark, R. A. F. (2008). Cutaneous Wound Healing. *New England Journal of Medicine*, *341*(10), 738–746. https://doi.org/10.1056/NEJM199909023411006

Slack, R. J., Macdonald, S. J. F., Roper, J. A., Jenkins, R. G., & Hatley, R. J. D. (2021). Emerging therapeutic opportunities for integrin inhibitors. *Nature Reviews. Drug Discovery*, 19. https://doi.org/10.1038/S41573-021-00284-4

Stern, E.P., & Denton, C.P. (2015). The Pathogenesis of Systemic Sclerosis. *Rheumatic Diseases Clinics of North America*, *41*(3), 367-382. https://doi.org/ 10.1016/J.RDC.2015.04.002

Su, B.-C., Hsu, P.-L., & Mo, F.-E. (2020). CCN1 triggers adaptive autophagy in cardiomyocytes to curb its apoptotic activities. *Journal of Cell Communication and Signaling*, *14*(1), 100. https://doi.org/10.1007/S12079-019-00534-6

Sun, G., Haginoya, K., Wu, Y., Chiba, Y., Nakanishi, T., Onuma, A., Sato, Y., Takigawa, M., Iinuma, K., & Tsuchiya, S. (2008). Connective tissue growth factor is overexpressed in muscles of human muscular dystrophy. *Journal of the Neurological Sciences*, *267*(1), 48–56. https://doi.org/10.1016/J.JNS.2007.09.043

Sun, Y., Zhang, J., Zhai, T., Li, H., Li, H., Huo, R., Shen, B., Wang, B., Chen, X., Li, N., & Teng, J. (2017). CCN1 promotes IL-1β production in keratinocytes by activating p38 MAPK signaling in psoriasis. *Scientific Reports*, *7*, 43310. https://doi.org/10.1038/SREP43310

Sun, Y., Zhang, J., Zhou, Z., Wu, P., Huo, R., Wang, B., Shen, Z., Li, H., Zhai, T., Shen, B., Chen, X., & Li, N. (2015). CCN1, a Pro-Inflammatory Factor, Aggravates Psoriasis Skin Lesions by Promoting Keratinocyte Activation. *Journal of Investigative Dermatology*, *135*(11), 2666–2675. https://doi.org/10.1038/JID.2015.231

Takahashi, K., & Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell*, *126*(4), 663–676. https://doi.org/10.1016/J.CELL.2006.07.024

Taroni, J. N., Greene, C. S., Martyanov, V., Wood, T. A., Christmann, R. B., Farber, H. W., Lafyatis, R. A., Denton, C. P., Hinchcliff, M. E., Pioli, P. A., Mahoney, J. M., & Whitfield, M. L. (2017). A novel multi-network approach reveals tissue-specific cellular modulators of fibrosis in systemic sclerosis. *Genome Medicine*, *9*(1), 27. https://doi.org/10.1186/S13073-017-0417-1

Thannickal, V. J., Lee, D. Y., White, E. S., Cui, Z., Larios, J. M., Chacon, R., Horowitz, J. C., Day, R. M., & Thomas, P. E. (2003). Myofibroblast differentiation by transforming growth factor-β1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *Journal of Biological Chemistry*, *278*(14), 12384–12389. https://doi.org/10.1074/jbc.M208544200

Thompson, A. E., & Pope, J. E. (2002). Increased prevalence of scleroderma in Southwestern Ontario: A cluster analysis. *Journal of Rheumatology*. https://doi.org/10.1016/j.jcorpfin.2017.03.011

Thompson, K., Murphy-Marshman, H., & Leask, A. (2014). ALK5 inhibition blocks TGFβ-induced CCN1 expression in human foreskin fibroblasts. *Journal of Cell Communication and Signaling*, *8*(1), 59. https://doi.org/10.1007/s12079-014-0229-7

Todorovicç, V., Chen, C.-C., Hay, N., & Lau, L. F. (2005). The matrix protein CCN1 (CYR61) induces apoptosis in fibroblasts. *Journal of Cell Biology*, *171*(3), 568. https://doi.org/10.1083/JCB.200504015

Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., & Brown, R. A. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nature Reviews Molecular Cell Biology*, *3*(5). https://doi.org/10.1038/nrm809

Toyama, T., Looney, A. P., Baker, B. M., Stawski, L., Haines, P., Simms, R., Szymaniak, A. D., Varelas, X., & Trojanowska, M. (2018). Therapeutic targeting of TAZ and YAP by dimethyl fumarate in systemic sclerosis fibrosis. *Journal of Investigative Dermatology*, *138*(1), 88. https://doi.org/10.1016/J.JID.2017.08.024

Tsang, M., & Leask, A. (2014). CCN2 is required for recruitment of Sox2-expressing cells during cutaneous tissue repair. *Journal of Cell Communication and Signaling*, *9*(4), 341–346. https://doi.org/10.1007/S12079-014-0245-7

Tsang, M., Quesnel, K., Vincent, K., Hutchenreuther, J., Postovit, L.-M., & Leask, A. (2019). Insights into Fibroblast Plasticity: Cellular Communication Network 2 Is Required for Activation of Cancer-Associated Fibroblasts in a Murine Model of Melanoma. *American Journal of Pathology*, *190*(1), 206–221. https://doi.org/10.1016/J.AJPATH.2019.09.006

van Roeyen, C. R. C., Eitner, F., Scholl, T., Boor, P., Kunter, U., Planque, N., Gröne, H. J., Bleau, A. M., Perbal, B., Ostendorf, T., & Floege, J. (2008). CCN3 is a novel endogenous PDGF-regulated inhibitor of glomerular cell proliferation. *Kidney International*, *73*(1), 86–94. https://doi.org/10.1038/SJ.KI.5002584

Varga, J., & Abraham, D. (2007). Systemic sclerosis: a prototypic multisystem fibrotic disorder. *Journal of Clinical Investigation*, *117*(3), 557–567. https://doi.org/10.1172/JCI31139

Verrecchia, F., & Mauviel, A. (2002). Transforming growth factor-β signaling through the Smad pathway: Role in extracellular matrix gene expression and regulation. In *Journal of Investigative Dermatology*. https://doi.org/10.1046/j.1523-1747.2002.01641.x

Walraven, M., & Hinz, B. (2018). Therapeutic approaches to control tissue repair and fibrosis: Extracellular matrix as a game changer. *Matrix Biology*, *71–72*, 205–224. https://doi.org/10.1016/J.MATBIO.2018.02.020

Wang, Y., Fan, P.-S., & Kahaleh, B. (2006). Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. *Arthritis & Rheumatology*, *54*(7), 2271–2279. https://doi.org/10.1002/ART.21948

Wynn, T. A. (2008). Cellular and molecular mechanisms of fibrosis. *Journal of Pathology*, *214*(2), 199–210. https://doi.org/10.1002/path.2277

Yanagihara, T., Chong, S. G., Gholiof, M., Lipson, K. E., Zhou, Q., Scallan, C., Upagupta, C., Tikkanen, J., Keshavjee, S., Ask, K., & Kolb, M. R. (2020). Connective-Tissue Growth Factor (CTGF/CCN2) Contributes to TGF-β1-Induced Lung Fibrosis. *BioRxiv*. https://doi.org/10.1101/2020.07.04.187492

Yao, Y., Li, B., Fu, C., Teng, G., Ma, G., & Liu, N. (2017). Anti-connective tissue growth factor detects and reduces plaque inflammation in early-stage carotid atherosclerotic lesions. *Nanomedicine: Nanotechnology, Biology and Medicine*, *13*(8), 2385–2394. https://doi.org/10.1016/J.NANO.2017.07.016

Zhong, L., Pope, M., Shen, Y., Hernandez, J. J., & Wu, L. (2019). Prevalence and incidence of systemic sclerosis: A systematic review and meta-analysis. *International Journal of Rheumatic Diseases*, *22*(12), 2096–2107. https://doi.org/10.1111/1756-185X.13716

Chapter 2

2 Yin/Yang expression of CCN family members: Transforming growth factor-β1, via ALK5/FAK/MEK1, induces CCN1 and CCN2, yet suppresses CCN3, expression in human dermal fibroblasts

2.1 Introduction

Fibrosis, as a pathology, is characterized by the excessive deposition of extracellular matrix (ECM), comprised principally of type I collagen, resulting in scar tissue that ultimately culminates in organ dysfunction and, often, death. Collectively, fibrosis and fibrosis-associated disorders account for ~45% of the deaths in the Western world (Borthwick et al., 2013). As a feature of end-stage disease, the contribution of fibrosis to human disease would be expected to rise due to an increasingly aging population. Fibrotic conditions of the skin include: hypertrophic scars that occur in response to burns or wounding, keloids, or scleroderma, in which skin (and internal organs) progressively scars resulting in dermatological effects such as itching, skin tightness and reduced mobility (Allanore et al., 2015; Schulz et al., 2018).

The effector cell of fibrosis is the myofibroblast, which responds to profibrotic cytokines such as TGFβ by increasing production, contraction, adhesion, and remodeling of the surrounding extracellular matrix (Leask & Abraham, 2004; Schulz et al., 2018). Initially it was believed, owing to its profound *in vitro* and *in vivo* effects and its potent upregulation in wound healing and fibrosis, that targeting TGFβ and its canonical signaling pathways would have profound palliative effects on fibrotic conditions. However, it is now widely appreciated that TGFβ is not an appropriate therapeutic target due to lack of efficacy relative to observed side effects. This problem was surmised *a priori*, leading to the search in the early 1990s for downstream effectors or cofactors of TGFβ that may have more selective profibrotic effects (Dammeier et al., 1998). Indeed, parallel studies examining: (1) non-canonical TGFβ signaling; (2) the mechanobiology of the profibrotic effector cell, the myofibroblast; and (3) collagen structure conclusively established that an enhanced, autocrine pro-adhesive signaling pathway was essential to promote and sustain fibrosis

(Liu et al., 2009; Shi-Wen et al., 2009; Thannickal et al., 2003; van der Slot et al., 2003; Walraven & Hinz, 2018). The convergence of these experimental approaches, namely those examining, on one hand, the identification of cofactors/downstream mediators of TGFβ and, on the other hand, an autocrine pro-adhesive signaling loop in promoting and sustaining fibrosis, have supported the hypothesis that targeting the cellular microenvironment may be an appropriate therapeutic approach (Huang & Ogawa, 2012; Leask, 2013a; Schulz et al., 2018). In particular, the CCN family of secreted pro-adhesive matricellular proteins are of interest (Leask, 2013b; Riser et al., 2015). CCN2, which is induced in fibroblasts by the potent profibrotic cytokine TGFβ, was hypothesized as being a mediator of fibrosis as early as the mid-1990s (Dammeier et al., 1998; Holmes et al., 2001; Igarashi et al., 1993). Indeed, conditional knockout strategies have shown CCN2 expression by fibroblasts is required for fibrosis in a variety of mouse models (Kinashi et al., 2017; Leask, 2013b; Liu et al., 2011; Makino et al., 2017; Petrosino et al., 2019). Conversely, CCN2 is not required for cutaneous tissue repair (Liu et al., 2014), emphasizing its selective profibrotic action and its potential utility as a specific anti-fibrotic target. Significantly, an anti-CCN2 antibody strategy (FG-3019) is currently undergoing Phase III trials for idiopathic pulmonary fibrosis (Raghu et al., 2016).

In addition to CCN2, the related CCN-family member CCN1 has context-specific profibrotic effects (Kurundkar et al., 2016). Thus, clinically, a more effective strategy might be to target both CCN1 and CCN2 simultaneously. In that regard, another member of the CCN family, CCN3, is reciprocally regulated by CCN2 in a model of diabetes (Riser et al., 2009, 2010), in glomerular cell proliferation (van Roeyen et al., 2008), and chondrocyte differentiation (Kawaki et al., 2008). Moreover, CCN3 protein has anti-fibrotic effects in a model of diabetic nephropathy (Riser et al., 2014). These data have led to the hypothesis that a high CCN2:CCN3 ratio drives fibrosis and that normalizing this ratio by adding CCN3 may have anti-fibrotic effects (Perbal, 2018; Riser et al., 2015). Furthermore, reciprocal regulation of CCN1 and CCN3 activities had also been previously discussed (Bleau et al., 2005). However, no studies have simultaneously examined the regulation of CCN1, CCN2, and CCN3, possibly because, until recently, the concept of all three proteins being members of the same family and therefore worthy of studying them simultaneously has not achieved widespread recognition (Perbal, 2018; Perbal et al., 2018).

To begin to address this conceptual deficit, I elected to add TGFβ1 to human dermal fibroblasts and simultaneously monitor the expression of CCN1, CCN2 and CCN3. Moreover, I used chemical signal transduction inhibitors to identify if a common pathway mediates TGFβ1's effects on CCN1, CCN2 and CCN3. Our data provide new and valuable insights into the reciprocal regulation of CCN proteins in fibroblasts and into the signaling mechanisms downstream of TGFβ1 in driving expression of key profibrotic mediators in fibroblasts.

2.2 Methods

2.2.1 Cell Culture

All cell culture experiments were performed using primary human foreskin fibroblasts obtained from healthy humans (American Type Culture Collection CRL2094) that were previously shown to differentiate into myofibroblasts in the presence of TGFβ1 or mechanical tension (Murphy-Marshman et al., 2017). Cells were cultured to passage 8 in high glucose DMEM (Invitrogen) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Cells were plated on tissue culture plastic at a density of 60000 cells/plate and grown overnight at 37°C in a humidifier containing 5% CO_2. At approximately 70% confluence, cells were serum starved by replacing high glucose DMEM with low glucose DMEM containing 0.5% FBS and 1% antibiotic-antimycotic solution. After serum starving overnight, cells were pre-treated with either DMSO (Sigma, 20 μM) or one of the following small molecule inhibitors: SB-431542 (Tocris, 10 μM), PF573228 (Sigma, 10 μM), (5Z)-7-oxozeaenol (Tocris, 400 nM), U0126 (Sigma, 30 μM), or Verteporfin (Sigma, 695 nM). TGFβ1 (R&D Systems, 0.3 μM) was added 30 minutes after inhibitors for either 6 hours (for RNA collection) or 24 hours (for protein collection).

2.2.2 Generation of CCN3-overexpressing Fibroblasts

Custom lentiviral particles were designed and obtained from Sigma-Aldrich. CCN3 overexpressing cells were generated using a CSTORFV Mission TRC3 Custom Human ORF Lentivirus (pLX317) containing a specifically designed *CCN3* expression vector. Transduction control cells were generated using an ORFBFPV Mission TRC3 ORF GFP Lentivirus. For transduction, human foreskin fibroblasts were incubated with viral particles

at a multiplicity of infection of 1.5 supplemented with 5 μg/mL Polybrene for 24 hours. Successfully transduced cells were selected for using puromycin as described by the manufacturer (Sigma-Aldrich). Surviving cells were cultured to passage 8.

2.2.3 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was obtained from treated cells after 6-hour treatments described above. TriZol extraction using phenol-chloroform was used to isolate RNA from cell lysates to be used for real-time PCR. RNA concentrations and integrity were measured via Nanodrop 2000 (Thermo Scientific). A total of 1 μg of RNA from each sample was reverse transcribed using qScript Supermix (QuantaBio), producing cDNA. SYBR green real-time PCR was then performed by combining cDNA (7 ng/well), SYBR master mix (Thermo Scientific) and gene specific primers. Signal changes were detected using a ViiA 7 Real-Time PCR System (Thermo Scientific). The following gene specific primers were purchased from Life Technologies for use in our experiment: *CCN1, CCN2, CCN3, EDN1, ITGA11* (Table 1). Samples were run in triplicate and expression values were standardized to control values from *ACTB* primers using the ΔΔCt method. Biological repeats are indicated. GraphPad Prism software was used to perform a one-way ANOVA with Tukey's post-hoc test to determine statistical significance.

Target	**Forward** (5' to 3')	**Reverse** (5' to 3')
ACTB	CCTCGCCTTTGCCGATCC	CGCGGCGATATCATCATCG
CCN1	CGGCTCCCTGTTTTTGGAAT	TTGAGCACTGGGACCATGAA
CCN2	GAGGAGTGGCTGTGTGACG	TCTTCCAGTCGGTAACCGC
CCN3	GTGCTACTGCCTGAGCCTAA	CTGTAAGCTGCAAGGGTAAGG
ITGA11	CTGTGGCCAGGGTTCACG	TGTAGCCAAAGAAGGCGGTC
EDN1	AGAAACAGTCTTAGGCGCTGA	TGGACTGGGAGTGGGTTTCT

Table 2-1. Primers used for qPCR.

2.2.4 Western Blot

Proteins were harvested using radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 100 mM Tris-HCl pH 7.4, 1% NP40, 0.1% SDS, 5 mM EDTA, 1X protease inhibitor cocktail) from cells after 24-hour treatment. Protein concentrations were approximated using a BCA protein assay kit (Thermo Scientific), according to instructions provided by the distributor. An equal amount of protein (50 μg) was added to each well of an SDS-PAGE polyacrylamide gel (5% stacking, 10% separating). Protein samples were resolved and then transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat skim milk in TBST (100mM Tris-HCl, pH 7.4, 0.01% Tween-20) for 1 hour, and then incubated with primary antibody overnight at 4°C in 5% milk solution. The following antibodies were used: anti-CCN1 (1:1000; abc102; Santa Cruz), anti-CCN2 (1:500; sc14939; Santa Cruz) and anti-ACTB (1:8000; A1978; Sigma-Aldrich). Anti-CCN3 antibody (dilution 1:2000), was used as described in the paper disclosing the generation of the antibody (Kyurkchiev et al., 2004). Membranes were washed thoroughly in TBST and then incubated with HRP-conjugated secondary antibody for 1 hour. Horseradish peroxidase-conjugated donkey anti-goat (705-036-147), donkey anti-rabbit (711-036-152) and donkey anti-mouse (715-035-150) were obtained from Jackson Immunoresearch Laboratories. Membranes were washed thoroughly and then exposed to SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 minutes before visualization using X-ray film.

2.3 Results

2.3.1 TGFβ1 induces *CCN1/CCN2* expression and CCN1/CCN2 protein levels yet suppresses *CCN3* expression in human dermal fibroblasts via ALK5

To begin to assess if, in human dermal fibroblasts, TGFβ1 affects CCN1, CCN2 and CCN3 regulation in a yin/yang fashion, we treated serum starved cells overnight and then cultured them with or without TGFβ1 for an additional 6 h (for RNA analysis) or 24 h (for protein analysis). RNA and protein were harvested. For our protein analysis, consistent with previous studies, we examined total cell extracts to capture CCN proteins in the process of being secreted through the Golgi (Chen et al., 2001). In our initial experiments, as a control,

we also treated cells in the presence or absence of an ALK5 inhibitor, as ALK5 is the TGFβ type I receptor for fibroblasts (Leask & Abraham, 2004). As expected, TGFβ1 induced *CCN1/CCN2* expression and CCN1/CCN2 protein levels, consistent with prior studies (Holmes et al., 2001; Thompson et al., 2014) (Figure 2-1). Moreover, consistent with prior studies, ALK5 inhibition blocked TGFβ1 induced *CCN1* expression and CCN1 protein levels in human dermal fibroblasts (Thompson et al., 2014) (Figure 2-1). Furthermore, TGFβ1 also induced *CCN2* expression and CCN2 protein levels in a fashion sensitive to ALK5 inhibition; conversely, TGFβ1 suppressed *CCN3* expression; this was also blocked by addition of ALK5 inhibitor (Figure 2-1).

However, to our surprise, CCN3 protein levels were undetectable in human dermal fibroblasts either in the presence or absence of added TGFβ1 (Figure 2-1). Conversely, CCN3 was readily detected in NCI-H295R cells, a pluripotent adrenocortical carcinoma cell line established by A.F. Gazdar and associates (Gazdar et al., 1990) from a carcinoma of the adrenal cortex. These cells are known to express abundant levels of CCN3 (Kyurkchiev et al., 2004), indicating the validity of our method of detecting CCN3 protein (Figure 2-1). That is, CCN3 protein does not appear to be basally expressed by proliferating human dermal fibroblasts [Previous studies have shown that low levels of intracellular CCN3 are produced in fibroblasts in culture; in growing cells the expression of CCN3 protein is quickly downregulated; (Scholz et al., 1996)]. Collectively however, these data are consistent with the idea that, at least at the mRNA level, TGFβ1 has opposing effects on *CCN1* and *CCN2* as compared to *CCN3* in human dermal fibroblasts and that this effect is mediated by ALK5.

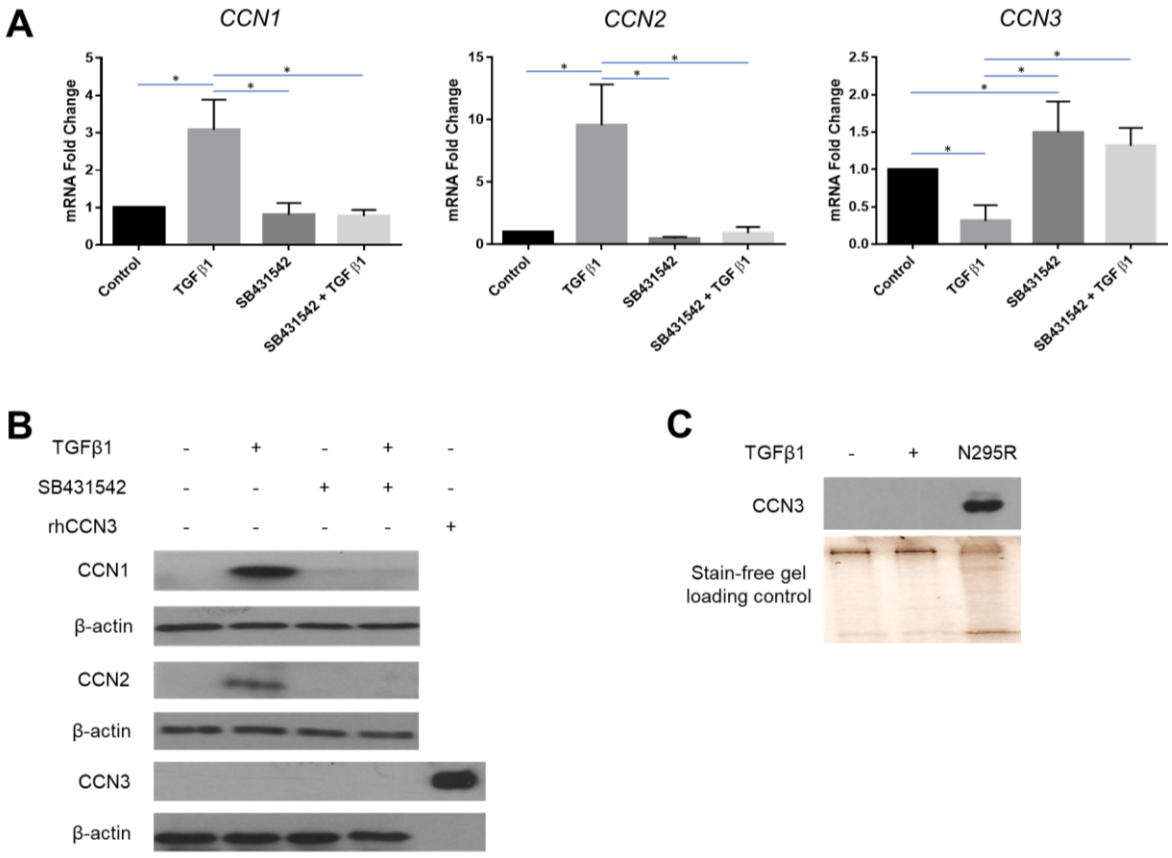

Figure 2-1. TGFβ1-responsiveness of CCN1, CCN2 and CCN3 is sensitive to ALK5 inhibition in human dermal fibroblasts.

Human dermal fibroblasts were cultured, and serum starved overnight, prior to pre-treatment with an ALK5 inhibitor, SB431542 (10 μM). After pre-treatment, cells were incubated either with or without TGFβ1 (0.3 μM). **(A)** Total RNA was extracted 6 hours after treatment and subjected to real-time PCR to detect changes in *CCN1*, *CCN2* and *CCN3* gene expression. Results are expressed as mean +/- SD. Statistical differences were determined using one-way ANOVA with Tukey's post-hoc test (*$p<0.05$, n=6). **(B)** Protein was extracted 24 hours after treatment and equal amounts of protein (50 μg) were resolved using SDS-PAGE. Western blot analysis was performed using antibodies against CCN1 (37 kDa), CCN2 (38 kDa) and CCN3 (49 kDa). ACTB (42 kDa) was used as a loading control. Representative blots are shown (n=3). **(C)** CCN3 protein is readily detected basally in NCI-H295R cancer cells but not in human dermal fibroblasts.

2.3.2 TGFβ1 induces CCN1 and CCN2 in human dermal fibroblasts via FAK

Prior data from our group and others has suggested that an autocrine pro-adhesive signaling loop operating through focal adhesion kinase (FAK) sustains fibrosis, and FAK appears to mediate TGFβ's profibrotic effects (Liu et al., 2007; Shi-Wen et al., 2012; Thannickal et al., 2003). To extend our current data, we then assessed whether addition of a FAK inhibitor could affect the ability of TGFβ1 to modulate expression of *CCN1*, *CCN2*, and *CCN3* in human dermal fibroblasts. We conducted experiments similar to those described above, however we cultured cells in the presence or absence of TGFβ1 and the presence or absence of the FAK inhibitor PF573228. We found that PF573228 blocked TGFβ1-induced *CCN1/CCN2* expression and CCN1/CCN2 protein levels in human dermal fibroblasts (Figure 2-2). TGFβ1-suppressed *CCN3* expression was insensitive to PF573228 (Figure 2-2). Given the profibrotic roles of CCN1 and CCN2, these results emphasize the critical, central role of adhesive signaling operating through FAK in mediating fibrogenic responses in response to TGFβ1.

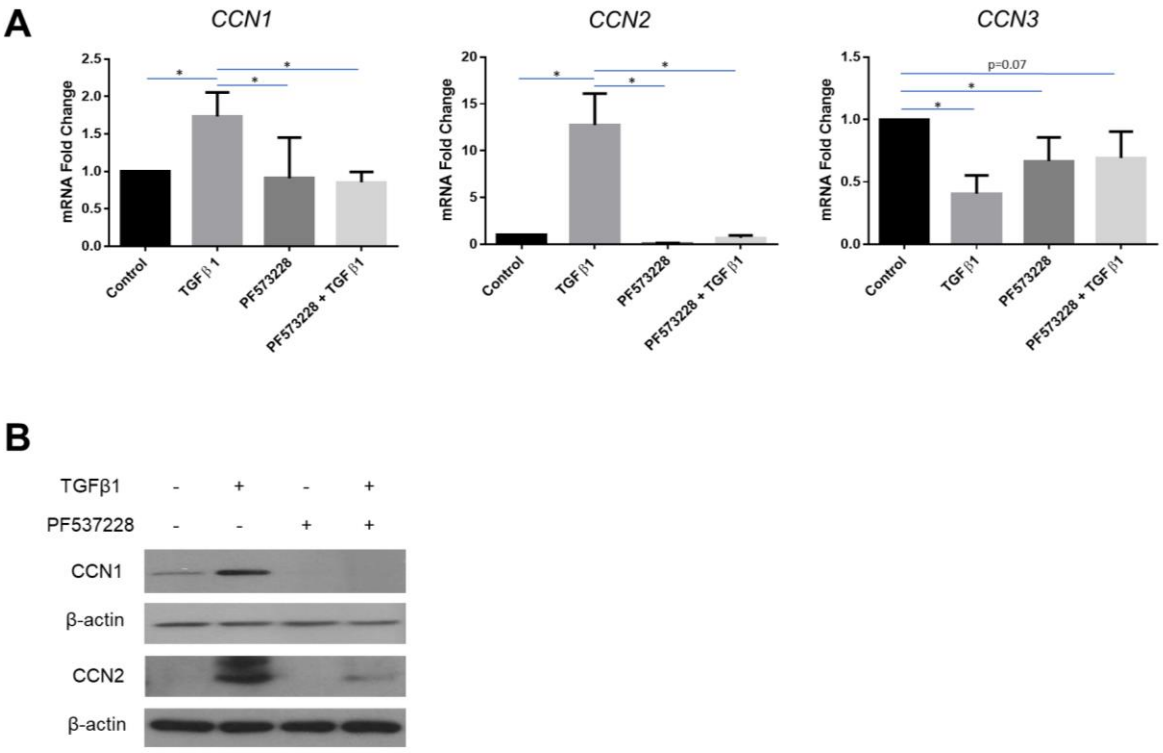

Figure 2-2. TGFβ1-responsiveness of CCN1 and CCN2 is sensitive to FAK inhibition in human dermal fibroblasts.

Human dermal fibroblasts were cultured, and serum starved overnight, prior to pre-treatment with a FAK inhibitor, PF573228 (10 μM). After pre-treatment, cells were incubated either with or without TGFβ1 (0.3 μM). **(A)** Total RNA was extracted 6 hours after treatment and subjected to real-time PCR to detect changes in *CCN1*, *CCN2* and *CCN3* gene expression. Results are expressed as mean +/- SD. Statistical differences were determined using one-way ANOVA with Tukey's post-hoc test (*$p<0.05$, n=4). **(B)** Protein was extracted 24 hours after treatment and equal amounts of protein (50 μg) were resolved using SDS-PAGE. Western blot analysis was performed using antibodies against CCN1 (37 kDa) and CCN2 (38 kDa). ACTB (42 kDa) was used as a loading control. Representative blots are shown (n=3).

2.3.3 TGFβ1 induces *CCN1/CCN2* expression and CCN1/CCN2 protein levels yet suppresses *CCN3* expression in human dermal fibroblasts via MEK1

Prior data from our group has indicated that TGFβ-induced *CCN2* expression occurs via MEK1/ERK (Leask et al., 2003). To assess if the ability of TGFβ1 to induce *CCN1* expression and suppress *CCN3* expression depended on MEK1, we repeated our studies in the presence or absence of the MEK1 inhibitor U0126. As anticipated, consistent with our prior reports, TGFβ1 induced *CCN2* expression and CCN2 protein levels in a manner that was impaired by U0126 (Leask et al., 2003) (Figure 2-3). Similarly, addition of U0126 significantly impaired the ability of TGFβ1 to induce *CCN1* expression and CCN1 protein levels in human dermal fibroblasts (Figure 2-3). Finally, addition of U0126 did not significantly impaired the ability of TGFβ1 to suppress *CCN3* expression in human dermal fibroblasts, although U0126 reduced baseline *CCN3* expression (Figure 2-3). These data emphasize the central role of MEK1 in mediating the fibrogenic responses of TGFβ1.

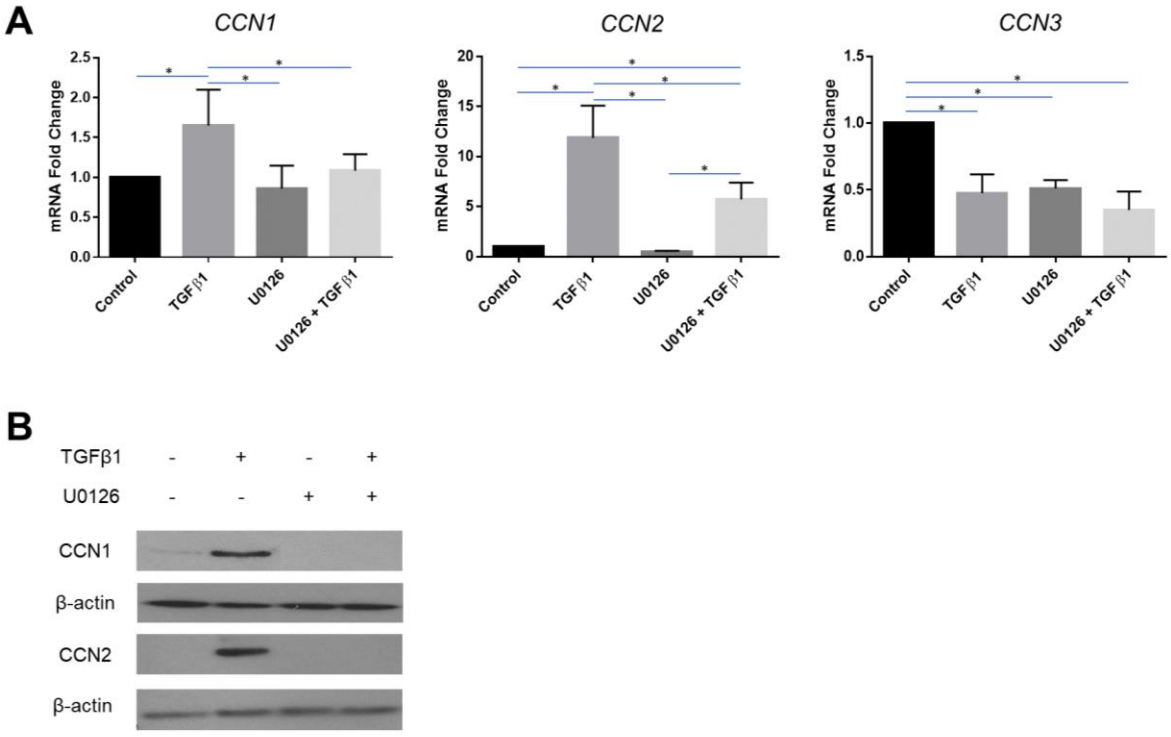

Figure 2-3. TGFβ1-responsiveness of CCN1 and CCN2 is sensitive to MEK1 inhibition in human dermal fibroblasts.

Human dermal fibroblasts were cultured, and serum starved overnight, prior to pre-treatment with a MEK1 inhibitor, U0126 (30 μM). After pre-treatment, cells were incubated either with or without TGFβ1 (0.3 μM). **(A)** Total RNA was extracted 6 hours after treatment and subjected to real-time PCR to detect changes in *CCN1*, *CCN2* and *CCN3* gene expression. Results are expressed as mean +/- SD. Statistical differences were determined using one-way ANOVA with Tukey's post-hoc test (*$p<0.05$, n=3). **(B)** Protein was extracted 24 hours after treatment and equal amounts of protein (50 μg) were resolved using SDS-PAGE. Western blot analysis was performed using antibodies against CCN1 (37 kDa) and CCN2 (38 kDa). ACTB (42 kDa) was used as a loading control. Representative blots are shown (n=3).

2.3.4 TGFβ1 induces CCN1 and CCN2 in human dermal fibroblasts via TAK1

Mitogen-activated protein kinase kinase kinase 7 (MAP3K7), also known as TAK1 (TGFβ-activated kinase 1), once activated, is an upstream activator of MKK/JNK and p38 by the phosphorylation and activation of MAP kinase kinases such as MAP2K1/MEK1, MAP2K3/MKK3, MAP2K6/MKK6 and MAP2K7/MKK7. TAK1 deletion blocks TGFβ-induced alpha-smooth muscle actin expression in mouse embryonic fibroblasts, and, in gingival fibroblasts, the TAK1 inhibitor (5Z)-7-Oxozeaenol blocks the ability of TGFβ1 to induce *CCN2* expression (Kuk et al., 2015; Shi-Wen et al., 2009). To extend these studies, we assessed the ability of (5Z)-7-Oxozeaenol to block the effect of TGFβ1 on *CCN1*, *CCN2*, and *CCN3* expression in human dermal fibroblasts.

We found that, when applied to human dermal fibroblasts, (5Z)-7-Oxozeaenol treatment impaired TGFβ1-induced *CCN1/CCN2* expression and CCN1/CCN2 protein levels; however, TGFβ1-suppressed *CCN3* expression was not significantly affected by (5Z)-7-Oxozeaenol (Figure 2-4). These data suggest that the mechanism by which TGFβ1 induces *CCN2* and *CCN1* is divergent from that mediating TGFβ1-suppressed *CCN3* expression.

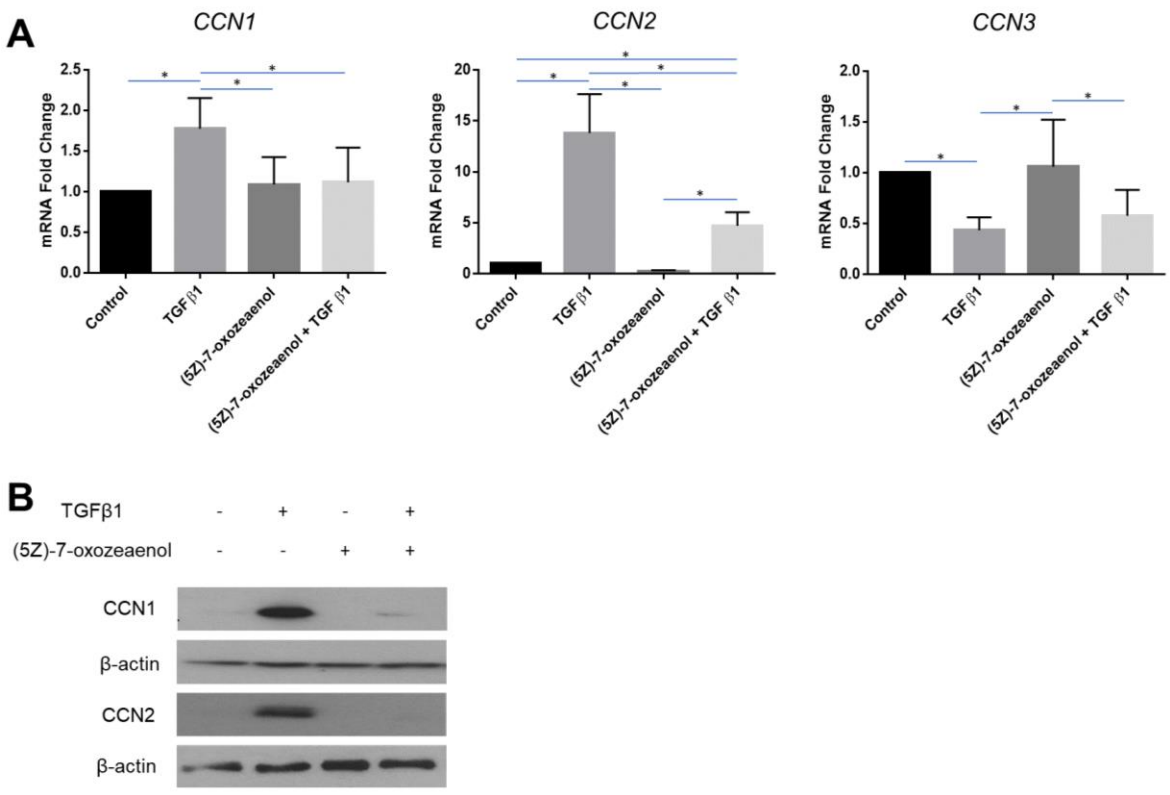

Figure 2-4. TGFβ1-responsiveness of CCN1 and CCN2, but not CCN3, is sensitive to TAK inhibition in human dermal fibroblasts.

Human dermal fibroblasts were cultured, and serum starved overnight, prior to pre-treatment with a TAK1 inhibitor, (5Z)-7-Oxozeaenol (400 nM). After pre-treatment, cells were incubated either with or without TGFβ1 (0.3 μM). **(A)** Total RNA was extracted 6 hours after treatment and subjected to real-time PCR to detect changes in *CCN1*, *CCN2* and *CCN3* gene expression. Results are expressed as mean +/- SD. Statistical differences were determined using one-way ANOVA with Tukey's post-hoc test (*$p<0.05$, n=6). **(B)** Protein was extracted 24 hours after treatment and equal amounts of protein (50 μg) were resolved using SDS-PAGE. Western blot analysis was performed using antibodies against CCN1 (37 kDa) and CCN2 (38 kDa). ACTB (42 kDa) was used as a loading control. Representative blots are shown (n=3).

2.3.5 TGFβ1 induces CCN1 and CCN2 in human dermal fibroblasts via YAP1

Previously, we showed that *CCN2* expression was promoted by the mechanosensitive oncogene YAP1 (Leask et al., 2003). Subsequently, the literature has used both *CCN1* and *CCN2* as stereotypical genes induced by the hippo/YAP1-TAZ pathway (Zhou et al., 2016). Given these observations and the potential importance of mechanotransduction in mediating pathological fibrosis, we ascertained if the selective YAP1 inhibitor verteporfin (Wang et al., 2016) could impair the effect of TGFβ1 on *CCN1*, *CCN2* and *CCN3* expression in human dermal fibroblasts.

In our experiments, exposure to verteporfin impaired the ability of human dermal fibroblasts to respond to TGFβ1 by increasing *CCN1/CCN2* expression and CCN1/CCN2 protein levels (Figure 2-5). However, YAP1 inhibition did not significantly affect the ability of TGFβ1 to suppress *CCN3* expression and, in fact, appeared to enhance the suppressive effect of TGFβ on *CCN3* expression ($p = 0.06$) (Figure 2-5). These data are consistent with the notions that verteporfin might be used to suppress the fibrogenic effects of TGFβ1 on fibroblasts; and that the mechanism underlying the ability of TGFβ1 to induce *CCN2* and *CCN1* differs from that through which TGFβ1 suppresses *CCN3* expression.

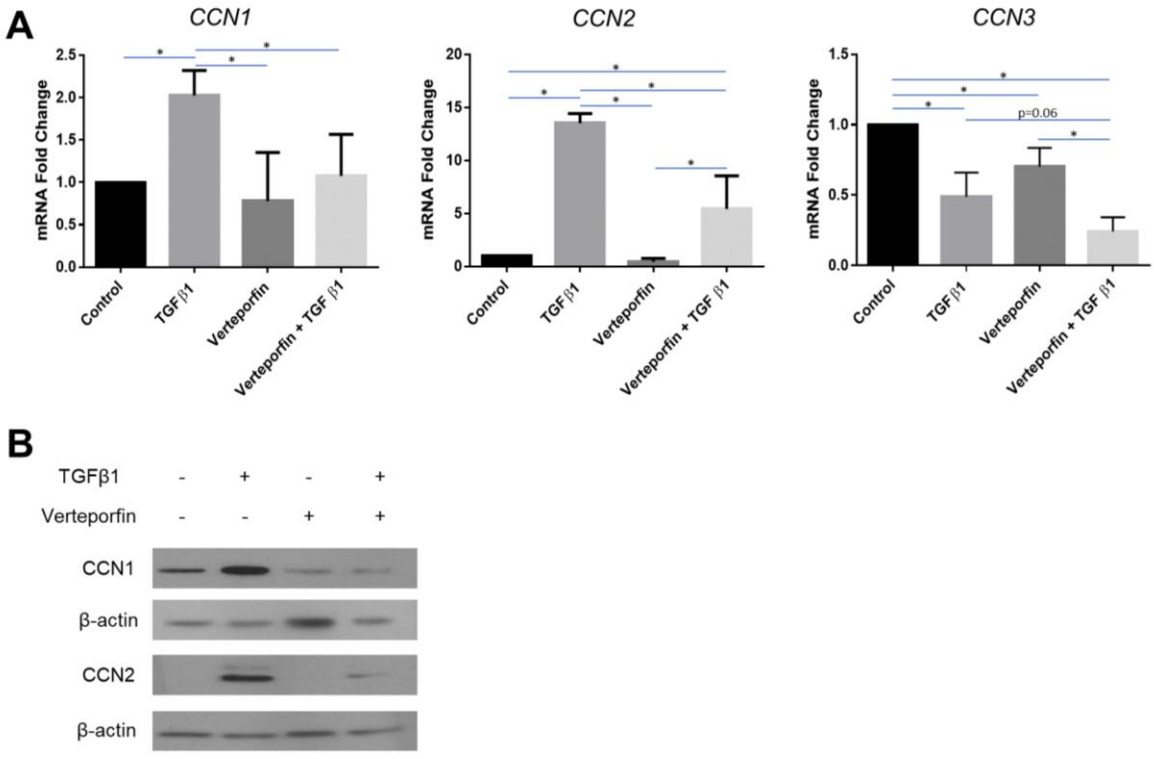

Figure 2-5. TGFβ1-responsiveness of CCN1 and CCN2, but not CCN3, is sensitive to YAP inhibition in human dermal fibroblasts.

Human dermal fibroblasts were cultured, and serum starved overnight, prior to pre-treatment with the YAP inhibitor verteporfin (695 nM). After pre-treatment, cells were incubated either with or without TGFβ1 (0.3 μM). **(A)** Total RNA was extracted 6 hours after treatment and subjected to real-time PCR to detect changes in *CCN1*, *CCN2* and *CCN3* gene expression. Results are expressed as mean +/- SD. Statistical differences were determined using one-way ANOVA with Tukey's post-hoc test (*$p<0.05$, n=6). **(B)** Protein was extracted 24 hours after treatment and equal amounts of protein (50 μg) were resolved using SDS-PAGE. Western blot analysis was performed using antibodies against CCN1 (37 kDa) and CCN2 (38 kDa). ACTB (42 kDa) was used as a loading control. Representative blots are shown (n=3).

2.3.6 Overexpression of CCN3 suppresses TGFβ1-induced CCN2 protein expression

As CCN3 has *in vivo* effects opposing that of CCN2, CCN3 has been proposed to be a potential anti-fibrotic treatment (Riser et al., 2015). To examine the possible mechanism underlying such an activity, we generated human dermal fibroblasts overexpressing CCN3. In these cells, TGFβ1 was able to induce CCN1 protein levels; however, the ability of TGFβ1 to induce CCN2 protein was significantly impaired (Figure 2-6A and 2-6B). Unsurprisingly, as: (a) in transduced fibroblasts *CCN3* was overexpressed under the control of the non-TGFβ-responsive CMV promoter; and (b) loss of CCN2 does not impair the ability of TGFβ to induce expression of fibrogenic mRNA in cultured dermal fibroblasts (Liu et al., 2011), overexpression of *CCN3* was not (a) suppressed by the addition of TGFβ1; and (b) able to impair the ability of TGFβ1 to induce the mRNA expression of the fibrogenic markers endothelin-1 (*EDN1*) or integrin alpha 11 (*ITGA11*) (Cipriani et al., 2015; Schulz et al., 2018; Shi-Wen et al., 2006) (Figure 2-6C).

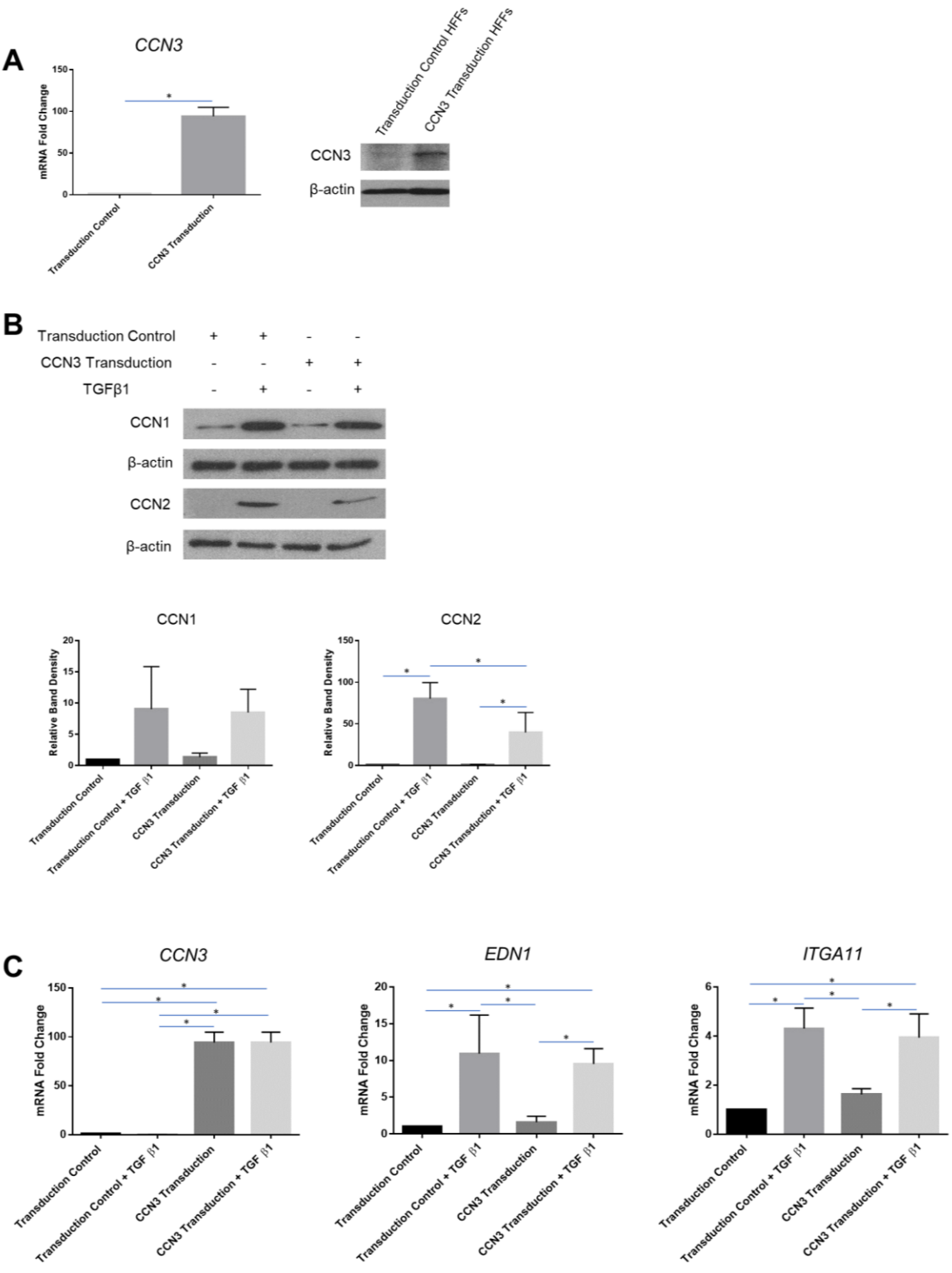
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Figure 2-6. CCN3 overexpression impairs TGFβ1-induced CCN2 protein expression.

Human dermal fibroblasts were transduced with lentiviral particles containing a *CCN3* expression vector, while transduction-control cells were transduced with lentiviral particles containing a control expression vector. **(A)** *CCN3* overexpression was confirmed via qPCR and Western blot analysis. CCN3-transduced cells show significantly more *CCN3* expression and CCN3 protein levels (49 kDa) than transduction-control cells. Statistical differences were determined using an unpaired t-test (*$p<0.05$, n=3). **(B)** Cells were serum starved overnight and then treated with or without TGFβ1 (0.3 μM). Protein was extracted 24 hours after treatment and equal amounts of protein (50 μg) were resolved using SDS-PAGE. Western blot analysis was performed using antibodies against CCN1 (37 kDa) and CCN2 (38 kDa). ACTB (42 kDa) was used as a loading control. Representative blots are shown (n=3). Densitometry analysis was performed using ImageJ software and statistical differences were determined using one-way ANOVA with Tukey's post-hoc test (*$p<0.05$). **(C)** Total RNA was extracted after treatment with or without TGFβ1 (0.3 μM) and subjected to real-time PCR to detect changes in *CCN3*, *EDN1*, and *ITGA11*. Results are expressed as mean +/- SD. Statistical differences were determined using one-way ANOVA with Tukey's post-hoc test (*$p<0.05$, n=3).

2.4 Discussion

The connective tissue microenvironment is being increasingly appreciated as playing a central role in promoting chronic disease (Hutchenreuther & Leask, 2018; Schulz et al., 2018). Specifically, the CCN family of matricellular proteins show altered expression patterns in connective tissue disease and are emerging targets for therapeutic intervention (Jun & Lau, 2011). CCN proteins share a similar structure, and have limited *in vitro* effects, making the development of relevant cell-based bioassays extremely difficult; consequently, it is necessary to study the functional role of CCN proteins *in vivo* (Leask, 2013b; Perbal, 2018). Of these, CCN2 (CTGF) is the most studied; indeed, anti-CCN2 antibodies are in clinical development (Raghu et al., 2016). Other CCN family members may have different functional roles *in vivo*, and indeed, may have opposing effects (Perbal, 2018). Of the other CCN proteins, CCN1 and CCN3 are the most studied; data published thus far suggest that, *in vivo*, CCN1 may have context-dependent profibrotic effects, whereas CCN3 may be anti-fibrotic (Kawaki et al., 2008; Riser et al., 2009, 2010, 2014; van Roeyen et al., 2008). However, until now, no study has simultaneously compared the regulation of CCN1, CCN2 and CCN3 in response to the profibrotic protein TGFβ1 in cultured dermal fibroblasts. Our data suggest that *CCN1/CCN2* expression and CCN1/CCN2 protein levels are induced by TGFβ1 via a similar pathway; conversely *CCN3* expression is reduced by TGFβ1 through a pathway that is divergent; that is, not involving MEK1, YAP1 or TAK1. These data are consistent with the general hypothesis that CCN1 and CCN2 are regulated in a yin/yang fashion opposite to CCN3 and with a hypothesis that, *in vivo*, restoring a CCN1/2:CCN3 balance may be of therapeutic value (Perbal, 2018; Riser et al., 2015). Similar to previous data showing that CCN2-deficient dermal fibroblasts retained TGFβ-responsiveness (Liu et al., 2011), overexpressing CCN3 in human dermal fibroblasts had no appreciable effect on the ability of TGFβ to induce mRNA expression of *EDN1* or *ITGA11*. It should be reiterated that, *in vivo*, loss or blockade of CCN2 expression severely impairs fibrogenesis, including myofibroblast differentiation, in a fashion that does not appear to involve canonical TGFβ signaling (Hutchenreuther et al., 2018; Kinashi et al., 2017; Leask, 2013b; Liu et al., 2011; Makino et al., 2017; Petrosino et al., 2019; Rebolledo et al., 2019). Since CCN proteins have limited *ex vivo* effects and instead act to integrate signaling emanating from multiple, distinct,

cellular sources (Perbal, 2018), direct testing of the potential anti-fibrotic role of CCN3 requires the use of animal models. However, these data suggest that CCN3 may work by blocking CCN2 expression in an as-yet-unidentified mechanism.

Our studies investigating the mechanism of how non-canonical TGFβ signaling activates CCN1 and CCN2 expression support the notion that FAK, ERK, TAK1 and YAP1 promote fibrogenic responses. The involvement of adhesive signaling via FAK and ERK (Li et al., 2018; Schulz et al., 2018; Shi-Wen et al., 2012) in promoting TGFβ signaling, and fibrosis is consistent with prior reports in other systems. YAP1 is known to activate genes in response to mechanotransduction; however, relatively few reports have examined the effect of verteporfin in blocking TGFβ's fibrogenic responses. Indeed, only two other reports have examined this question. Specifically, verteporfin was shown to reduce TGFβ responses in NRK renal cells and in conjunctival fibroblasts (Futakuchi et al., 2018; Szeto et al., 2016). This result is of potential long-term clinical application as verteporfin is in clinical use for macular degeneration (Gibault et al., 2016).

Collectively, our data provide new and valuable insights into the coordinated and opposite regulation of the key CCN family members CCN1, CCN2 and CCN3 in human dermal fibroblasts and are consistent with the hypothesis that alterations in the regulation of CCN proteins in response to fibrogenic stimuli may be important in driving fibrogenic responses. Our results showing that overexpression of CCN3 in fibroblasts reduces the ability of TGFβ to induce CCN2 protein expression are consistent with that notion and support the idea that CCN3-based peptides may function by inhibiting CCN2 activity. Our results are also consistent with the notion that TGFβ induces fibrogenic responses in fibroblasts via non-canonical pro-adhesive/mechanotransductive pathways and that targeting this pathway, for example, by using verteporfin, may be of value.

2.5 References

Allanore, Y., Simms, R., Distler, O., Trojanowska, M., Pope, J., Denton, C. P., & Varga, J. (2015). Systemic sclerosis. *Nature Reviews Disease Primers*, *1*(15002), 1–21. https://doi.org/10.1038/nrdp.2015.2

Bleau, A. M., Planque, N., & Perbal, B. (2005). CCN proteins and cancer: two to tango. *Frontiers in Bioscience*, *10*, 998–1009. https://doi.org/10.2741/1594

Borthwick, L. A., Wynn, T. A., & Fisher, A. J. (2013). Cytokine mediated tissue fibrosis. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1832*(7), 1049–1060. https://doi.org/10.1016/j.bbadis.2012.09.014

Chen, Y., Segarini, P., Raoufi, F., Bradham, D., & Leask, A. (2001). Connective Tissue Growth Factor Is Secreted through the Golgi and Is Degraded in the Endosome. *Experimental Cell Research*, *271*(1), 109–117. https://doi.org/10.1006/EXCR.2001.5364

Cipriani, P., Benedetto, P. di, Ruscitti, P., Verzella, D., Fischietti, M., Zazzeroni, F., Liakouli, V., Carubbi, F., Berardicurti, O., Alesse, E., & Giacomelli, R. (2015). Macitentan inhibits the transforming growth factor-β profibrotic action, blocking the signaling mediated by the ETR/TβRI complex in systemic sclerosis dermal fibroblasts. *Arthritis Research & Therapy*, *17*(1), 1–9. https://doi.org/10.1186/S13075-015-0754-7

Dammeier, J., Brauchle, M., Falk, W., Grotendorst, G. R., & Werner, S. (1998). Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? *International Journal of Biochemistry & Cell Biology*, *30*(8), 909–922. https://doi.org/10.1016/S1357-2725(98)00046-6

Futakuchi, A., Inoue, T., Wei, F.-Y., Inoue-Mochita, M., Fujimoto, T., Tomizawa, K., & Tanihara, H. (2018). YAP/TAZ Are Essential for TGF-b2-Mediated Conjunctival Fibrosis. *Investigative Opthalmology and Visual Science*, *59*, 3069–3078. https://doi.org/10.1167/iovs.18-24258

Gazdar, A. F., Oie, H. K., Shackleton, C. H., Chen, T. R., Triche, T. J., Myers, C. E., Chrousos, G. P., Brennan, M. F., Stein, C. A., & la Rocca, R. v. (1990). Establishment and Characterization of a Human Adrenocortical Carcinoma Cell Line That Expresses Multiple Pathways of Steroid Biosynthesis. *Cancer Research*, *50*(17), 5488–5496.

Gibault, F., Corvaisier, M., Bailly, F., Huet, G., Melnyk, P., & Cotelle, P. (2016). Non-Photoinduced Biological Properties of Verteporfin. *Current Medicinal Chemistry*, *23*(11), 1171–1184. https://doi.org/10.2174/0929867323666160316125048

Holmes, A., Abraham, D. J., Sa, S., Shiwen, X., Black, C. M., & Leask, A. (2001). CTGF and SMADs, Maintenance of Scleroderma Phenotype Is Independent of SMAD Signaling. *Journal of Biological Chemistry*, *276*(14), 10594–10601. https://doi.org/10.1074/jbc.M010149200

Huang, C., & Ogawa, R. (2012). Fibroproliferative disorders and their mechanobiology. *Connective Tissue Research*, *53*(3), 187–196. https://doi.org/10.3109/03008207.2011.642035

Hutchenreuther, J., & Leask, A. (2018). Why target the tumor stroma in melanoma? *Journal of Cell Communication and Signaling*, *12*(1), 113. https://doi.org/10.1007/S12079-017-0419-1

Hutchenreuther, J., Vincent, K., Norley, C., Racanelli, M., Gruber, S. B., Johnson, T. M., Fullen, D. R., Raskin, L., Perbal, B., Holdsworth, D. W., Postovit, L. M., & Leask, A. (2018). Activation of cancer-associated fibroblasts is required for tumor

neovascularization in a murine model of melanoma. *Matrix Biology*, *74*, 52–61. https://doi.org/10.1016/J.MATBIO.2018.06.003

Igarashi, A., Okochi, H., Bradham, D. M., & Grotendorst, G. R. (1993). Regulation of Connective Tissue Growth Factor Gene Expression in Human Skin Fibroblasts and During Wound Repair. In *Molecular Biology of the Cell* (Vol. 4).

Jun, J. il, & Lau, L. F. (2011). Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. In *Nature Reviews Drug Discovery*. https://doi.org/10.1038/nrd3599

Kawaki, H., Kubota, S., Suzuki, A., Lazar, N., Yamada, T., Matsumura, T., Ohgawara, T., Maeda, T., Perbal, B., Lyons, K. M., & Takigawa, M. (2008). Cooperative Regulation of Chondrocyte Differentiation by CCN2 and CCN3 Shown by a Comprehensive Analysis of the CCN Family Proteins in Cartilage. *Journal of Bone and Mineral Research*, *23*, 1751–1764. https://doi.org/10.1359/JBMR.080615

Kinashi, H., Falke, L. L., Nguyen, T. Q., Bovenschen, N., Aten, J., Leask, A., Ito, Y., & Goldschmeding, R. (2017). Connective tissue growth factor regulates fibrosis-associated renal lymphangiogenesis. *Kidney International*, *92*(4), 850–863. https://doi.org/10.1016/J.KINT.2017.03.029

Kuk, H., Hutchenreuther, J., Murphy-Marshman, H., Carter, D., & Leask, A. (2015). 5Z-7-Oxozeanol Inhibits the Effects of TGFβ1 on Human Gingival Fibroblasts. *PLoS ONE*, *10*(4), e0123689. https://doi.org/10.1371/JOURNAL.PONE.0123689

Kurundkar, A. R., Kurundkar, D., Rangarajan, S., Locy, M. L., Zhou, Y., Liu, R.-M., Zmijewski, J., & Thannickal, V. J. (2016). The matricellular protein CCN1 enhances TGF-b1/ SMAD3-dependent profibrotic signaling in fibroblasts and contributes to fibrogenic responses to lung injury. *FASEB Journal*, *30*(6), 2135–2150. https://doi.org/10.1096/fj.201500173

Kyurkchiev, S., Yeger, H., Bleau, A.-M., & Perbal, B. (2004). Potential cellular conformations of the CCN3(NOV) protein. *Cell Communication & Signaling*, *2*, 9. https://doi.org/10.1186/1478-811X-2-9

Leask, A. (2013a). Integrin β1: A Mechanosignaling Sensor Essential for Connective Tissue Deposition by Fibroblasts. *Advances in Wound Care*, *2*(4), 160. https://doi.org/10.1089/WOUND.2012.0365

Leask, A. (2013b). CCN2: a novel, specific and valid target for anti-fibrotic drug intervention. *Expert Opinion on Therapeutic Targets*, *17*(9), 1067–1071. https://doi.org/10.1517/14728222.2013.812074

Leask, A., & Abraham, D. (2004). TGF-β signaling and the fibrotic response. *FASEB Journal*, *18*(7), 816–827. https://doi.org/10.1096/fj.03-1273rev

Leask, A., Holmes, A., Black, C. M., & Abraham, D. J. (2003). Connective tissue growth factor gene regulation: Requirements for its induction by transforming growth factor-β2 in fibroblasts. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M210366200

Li, S., Liu, J., Tan, J., Li, L., Kaltreider, M. J., Zhao, J., Kass, D. J., Shang, D., & Zhao, Y. (2018). Inhibition of Raf1 ameliorates bleomycin-induced pulmonary fibrosis through attenuation of TGF-1 signaling. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *315*, 241–247. https://doi.org/10.1152/ajplung.00093.2018.-Idiopathic

Liu, S., Kapoor, M., Denton, C. P., Abraham, D. J., & Leask, A. (2009). Loss of 1 Integrin in Mouse Fibroblasts Results in Resistance to Skin Scleroderma in a Mouse Model. *Arthritis & Rheumatology*, *60*(9), 2817–2821. https://doi.org/10.1002/art.24801

Liu, S., Shi-Wen, X., Abraham, D. J., & Leask, A. (2011). CCN2 is required for bleomycin-induced skin fibrosis in mice. *Arthritis & Rheumatology*, *63*(1), 239–246. https://doi.org/10.1002/art.30074

Liu, S., Shi-wen, X., Kennedy, L., Pala, D., Chen, Y., Eastwood, M., Carter, D. E., Black, C. M., Abraham, D. J., & Leask, A. (2007). FAK Is Required for TGFβ-induced JNK Phosphorylation in Fibroblasts: Implications for Acquisition of a Matrix-remodeling Phenotype. *Molecular Biology of the Cell*, *18*(6), 2169. https://doi.org/10.1091/MBC.E06-12-1121

Liu, S., Thompson, K., & Leask, A. (2014). CCN2 expression by fibroblasts is not required for cutaneous tissue repair. *Wound Repair and Regeneration*, *22*(1), 119–124. https://doi.org/10.1111/wrr.12131

Makino, K., Makino, T., Stawski, L., Lipson, K. E., Leask, A., & Trojanowska, M. (2017). Anti-connective tissue growth factor (CTGF/CCN2) monoclonal antibody attenuates skin fibrosis in mice models of systemic sclerosis. *Arthritis Research & Therapy*, *19*(1), 134. https://doi.org/10.1186/s13075-017-1356-3

Murphy-Marshman, H., Quensel, K., Xu, S. W., Barnfield, R., Kelly, J., Peidl, A., Stratton, R. J., & Leask, A. (2017). Antioxidants and NOX1/NOX4 inhibition blocks TGFβ1-induced CCN2 and α-SMA expression in dermal and gingival fibroblasts. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0186740

Perbal, B. (2018). The concept of the CCN protein family revisited: a centralized coordination network. *Journal of Cell Communication and Signaling*, *12*(1), 3. https://doi.org/10.1007/S12079-018-0455-5

Perbal, B., Tweedie, S., & Bruford, E. (2018). The official unified nomenclature adopted by the HGNC calls for the use of the acronyms, CCN1–6, and discontinuation in the use of CYR61, CTGF, NOV and WISP 1–3 respectively. *Journal of Cell Communication and Signaling*, *12*(4), 625–629. https://doi.org/10.1007/s12079-018-0491-1

Petrosino, J. M., Leask, A., & Accornero, F. (2019). Genetic manipulation of CCN2/CTGF unveils cell-specific ECM-remodeling effects in injured skeletal muscle. *FASEB Journal*, *33*(2), 2047–2057. https://doi.org/10.1096/fj.201800622RR

Raghu, G., Scholand, M. B., de Andrade, J., Lancaster, L., Mageto, Y., Goldin, J., Brown, K. K., Flaherty, K. R., Wencel, M., Wanger, J., Neff, T., Valone, F., Stauffer, J., & Porter, S. (2016). FG-3019 anti-connective tissue growth factor monoclonal antibody: Results of an open-label clinical trial in idiopathic pulmonary fibrosis.

European Respiratory Journal, *47*, 1481–1491. https://doi.org/10.1183/13993003.01030-2015

Rebolledo, D. L., González, D., Faundez-Contreras, J., Contreras, O., Vio, C. P., Murphy-Ullrich, J. E., Lipson, K. E., & Brandan, E. (2019). Denervation-induced skeletal muscle fibrosis is mediated by CTGF/CCN2 independently of TGF-β. *Matrix Biology*, *82*, 20–37. https://doi.org/10.1016/J.MATBIO.2019.01.002

Riser, B. L., Barnes, J. L., & Varani, J. (2015). Balanced regulation of the CCN family of matricellular proteins: a novel approach to the prevention and treatment of fibrosis and cancer. *Journal of Cell Communication and Signaling*, *9*(4), 327. https://doi.org/10.1007/s12079-015-0309-3

Riser, B. L., Najmabadi, F., Garchow, K., Barnes, J. L., Peterson, D. R., & Sukowski, E. J. (2014). Treatment with the Matricellular Protein CCN3 Blocks and/or Reverses Fibrosis Development in Obesity with Diabetic Nephropathy. *American Journal of Pathology*, *184*(11), 2908–2921. https://doi.org/10.1016/j.ajpath.2014.07.009

Riser, B. L., Najmabadi, F., Perbal, B., Peterson, D. R., Rambow, J. A., Riser, M. L., Sukowski, E., Yeger, H., & Riser, S. C. (2009). CCN3 (NOV) is a negative regulator of CCN2 (CTGF) and a novel endogenous inhibitor of the fibrotic pathway in an in vitro model of renal disease. *American Journal of Pathology*, *174*(5), 1725–1734. https://doi.org/10.2353/ajpath.2009.080241

Riser, B. L., Najmabadi, F., Perbal, B., Rambow, J. A., Riser, M. L., Sukowski, E., Yeger, H., Riser, S. C., & Peterson, D. R. (2010). CCN3/CCN2 regulation and the fibrosis of diabetic renal disease. *Journal of Cell Communication and Signaling*, *4*(1), 39. https://doi.org/10.1007/s12079-010-0085-z

Scholz, G., Martinerie, C., Perbal, B., & Hanafusa, H. (1996). Transcriptional Down Regulation of the nov Proto-Oncogene in Fibroblasts Transformed by p60 v-src. In *Molecular and Cellular Biology*, *16*(2), 481-486. https://journals.asm.org/journal/mcb

Schulz, J. N., Plomann, M., Sengle, G., Gullberg, D., Krieg, T., & Eckes, B. (2018). New developments on skin fibrosis - Essential signals emanating from the extracellular matrix for the control of myofibroblasts. *Matrix Biology*, *68–69*, 522–532. https://doi.org/10.1016/J.MATBIO.2018.01.025

Shi-Wen, X., Parapuram, S. K., Pala, D., Chen, Y., Carter, D. E., Eastwood, M., Denton, C. P., Abraham, D. J., & Leask, A. (2009). Requirement of Transforming Growth Factor-Activated Kinase 1 for Transforming Growth Factor-Induced-Smooth Muscle Actin Expression and Extracellular Matrix Contraction in Fibroblasts. *Arthritis & Rheumatology*, *60*(1), 234–241. https://doi.org/10.1002/art.24223

Shi-Wen, X., Rodríguez-Pascual, F., Lamas, S., Holmes, A., Howat, S., Pearson, J. D., Dashwood, M. R., du Bois, R. M., Denton, C. P., Black, C. M., Abraham, D. J., & Leask, A. (2006). Constitutive ALK5-Independent c-Jun N-Terminal Kinase Activation Contributes to Endothelin-1 Overexpression in Pulmonary Fibrosis: Evidence of an Autocrine Endothelin Loop Operating through the Endothelin A and B Receptors. *Molecular and Cellular Biology*, *26*(14), 5518–5527. https://doi.org/10.1128/MCB.00625-06

Shi-Wen, X., Thompson, K., Khan, K., Liu, S., Murphy-Marshman, H., Baron, M., Denton, C. P., Leask, A., & Abraham, D. J. (2012). Focal adhesion kinase and reactive oxygen species contribute to the persistent fibrotic phenotype of lesional scleroderma fibroblasts. *Rheumatology*. https://doi.org/10.1093/rheumatology/kes234

Szeto, S. G., Narimatsu, M., Lu, M., He, X., Sidiqi, A. M., Tolosa, M. F., Chan, L., Freitas, K. de, Bialik, J. F., Majumder, S., Boo, S., Hinz, B., Dan, Q., Advani, A., John, R., Wrana, J. L., Kapus, A., & Yuen, D. A. (2016). YAP/TAZ Are Mechanoregulators of TGF-β-Smad Signaling and Renal Fibrogenesis. *Journal of the American Society of Nephrology*, *27*(10), 3117–3128. https://doi.org/10.1681/ASN.2015050499

Thannickal, V. J., Lee, D. Y., White, E. S., Cui, Z., Larios, J. M., Chacon, R., Horowitz, J. C., Day, R. M., & Thomas, P. E. (2003). Myofibroblast differentiation by transforming growth factor-β1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *Journal of Biological Chemistry*, *278*(14), 12384–12389. https://doi.org/10.1074/jbc.M208544200

Thompson, K., Murphy-Marshman, H., & Leask, A. (2014). ALK5 inhibition blocks TGFβ-induced CCN1 expression in human foreskin fibroblasts. *Journal of Cell Communication and Signaling*, *8*(1), 59. https://doi.org/10.1007/s12079-014-0229-7

van der Slot, A. J., Zuurmond, A.-M., Bardoel, A. F. J., Wijmenga, C., Pruijs, H. E. H., Sillence, D. O., Brinckmann, J., Abraham, D. J., Black, C. M., Verzijl, N., DeGroot, J., Hanemaaijer, R., TeKoppele, J. M., Huizinga, T. W. J., & Bank, R. A. (2003). Identification of PLOD2 as Telopeptide Lysyl Hydroxylase, an Important Enzyme in Fibrosis *. *Journal of Biological Chemistry*, *278*(42), 40967–40972. https://doi.org/10.1074/JBC.M307380200

van Roeyen, C. R. C., Eitner, F., Scholl, T., Boor, P., Kunter, U., Planque, N., Gröne, H. J., Bleau, A. M., Perbal, B., Ostendorf, T., & Floege, J. (2008). CCN3 is a novel endogenous PDGF-regulated inhibitor of glomerular cell proliferation. *Kidney International*, *73*(1), 86–94. https://doi.org/10.1038/SJ.KI.5002584

Walraven, M., & Hinz, B. (2018). Therapeutic approaches to control tissue repair and fibrosis: Extracellular matrix as a game changer. *Matrix Biology*, *71–72*, 205–224. https://doi.org/10.1016/J.MATBIO.2018.02.020

Wang, C., Zhu, X., Feng, W., Yu, Y., Jeong, K., Guo, W., Lu, Y., & Mills, G. B. (2016). Verteporfin inhibits YAP function through up-regulating 14-3-3σ sequestering YAP in the cytoplasm. *American Journal of Cancer Research*, *6*(1), 27. /pmc/articles/PMC4759394/

Zhou, Y., Huang, T., L Cheng, A. S., Yu, J., Kang, W., Fai To, K., & Chi-shing Cho, W. (2016). The TEAD Family and Its Oncogenic Role in Promoting Tumorigenesis. *International Journal of Molecular Sciences*, *17*(1), 138. https://doi.org/10.3390/ijms17010138

Chapter 3

3 Therapeutic peptides based on CCN3 prevent fibrosis in a mouse model of systemic sclerosis

3.1 Introduction

Fibrotic conditions, such as the often-fatal disease systemic sclerosis (SSc), are characterized by a loss of extracellular matrix (ECM) homeostasis and dysregulation of the normal wound healing process, leading to the excessive deposition of ECM components, such as collagen and fibronectin, resulting in pathological scarring that can result in organ failure and death (Leask, 2015; Schulz et al., 2018). In patients with SSc, a number of tissues are affected by fibrosis. In its diffuse form, patients may develop excessive fibrosis in their skin, liver, kidney, oral cavity, and lungs. Symptoms can range in severity and often include ulcers, tight and itchy skin, loss of mobility, and pulmonary hypertension (Allanore et al., 2015; Belloli et al., 2008; Czirják et al., 2008; Lóránd et al., 2014). Currently, there is no approved treatment available for SSc patients. This issue is due, in part, to the lack of suitable, specific targets, as well as a lack of unbiased, reliable, non-invasive methods for tracking disease progression (Varga & Abraham, 2007). These observations highlight the need to characterize the disease, as well as determine a suitable target on which to base therapeutic intervention.

In fibrotic lesions, resident fibroblasts within the ECM become activated and differentiate into myofibroblasts (Hinz, 2010). A critical indicator that distinguishes clinically involved from clinically uninvolved tissue in SSc patients is the presence of myofibroblasts (Rajkumar et al., 2005). Recent evidence suggests that the myofibroblast population in fibrotic lesions are derived, at least in part, from collagen-lineage resident dermal fibroblasts that become activated through a progenitor cell-like intermediate (Liu et al., 2014; Tsang et al., 2019). These myofibroblasts intrinsically possess abnormally active adhesive signaling and will excessively synthesize, adhere to and contract ECM. Activated myofibroblasts are characterized by excessive collagen secretion and overexpression of the highly contractile protein α-smooth muscle actin (αSMA) which contributes to the increased mechanical tension of fibrotic tissue (Hinz, 2015). Increased mechanical tension

in the ECM can lead to further activation of resident fibroblasts into active myofibroblasts (Hinz, 2019). Thus, fibrosis is maintained by an autocrine, pro-adhesive signaling loop. Broad targeting of this pathway may have deleterious effects on normal physiology. Thus, targeting specific downstream mediators of this feedback loop may represent a viable anti-fibrotic therapy.

A growing body of evidence has shown that the Cellular Communication Network (CCN) family of matricellular proteins is an important contributor to myofibroblast activation, and sustained adhesive and contractile signaling in fibrosis (Leask, 2013; Leask & Abraham, 2006; Perbal, 2004). The CCN family comprises six secreted proteins containing highly similar modular structure. Although originally thought to act as traditional growth factors, it is now known that the CCN proteins primarily function by modulating the signaling of other ECM molecules, such as transforming growth factor β (TGFβ) (Leask & Abraham, 2004). CCN proteins are known to play a role in development, inflammation, tissue repair, and a broad range of pathological processes including fibrosis and cancer (Chen & Lau, 2009; Perbal, 2013). The activity of CCN proteins is complex and highly context-dependent, with varying responses depending on the biological system. Thus, it is essential to study CCN protein function *in vivo*.

The most well studied member of the CCN family, CCN2 (formerly CTGF) is induced in differentiated myofibroblasts during wound healing and is overexpressed in fibrotic disease, as illustrated by the presence of CCN2 in the blister fluid of SSc patients (Holmes et al., 2011; Leask, 2013). CCN2 is a key, selective downstream mediator of fibrosis in multiple fibrotic models, including cardiac, renal and hepatic fibrosis (Dorn et al., 2018; Parapuram et al., 2015; Phanish et al., 2010; Rachfal & Brigstock, 2003). In fact, CCN2 expression by fibroblasts is required for fibrosis development in bleomycin-induced dermal fibrosis (Liu et al., 2011). Furthermore, CCN2 is required for recruitment and activation of collagen-lineage resident dermal fibroblasts into αSMA-expressing myofibroblasts (Liu et al., 2014, Tsang et al., 2019).

Another member of the CCN family, CCN1 (formerly CYR61), has structural and functional similarities to CCN2 *in vitro* and *in vivo* (Jun & Lau, 2011; Perbal, 2018).

Although the role of CCN1 in pathological processes such as fibrosis is less known, CCN1 has been shown to play a context-dependent role in promoting inflammatory responses, including in SSc (Kubota & Takigawa, 2007; Quesnel, 2019). CCN1 is also induced by pro-fibrotic stimuli *in vitro* (Thompson et al., 2014). Like CCN2, CCN1 expression by fibroblasts is required for bleomycin-induced dermal fibrosis, and likely plays a crucial role in collagen deposition and accumulation in the dermis (Quesnel et al., 2019). Altogether, there is ample evidence that CCN1 and CCN2 represent viable anti-fibrotic targets.

Recent work has shown that another member of the CCN family, CCN3, is reciprocally regulated to CCN1 and CCN2 in several cell types, and also exhibits opposing functional behaviour (Abd El Kader et al., 2012; Lemaire et al., 2010; Perbal, 2018; Riser et al., 2009). Furthermore, treatment with recombinant human CCN3 (rhCCN3) was able to block and reverse fibrosis in a mouse model of diabetic nephropathy (Riser et al., 2014). Taken together, these results suggest that CCN3 may act as an endogenous antagonist of CCN1 and CCN2 activity. These discoveries led to the development of a proprietary CCN3-based peptide (BLR-200) based on regions of CCN3 responsible for its anti-fibrotic effects *in vivo*. Initial studies have shown that BLR-200 can block mesangial cell adhesion to CCN2 and inhibit the ability of pro-fibrotic agents to provoke collagen expression in dermal fibroblasts *in vitro* (U.S. Patent No. 9114112B2, 2015). Thus, BLR-200 may represent a novel therapeutic approach for treatment of dermal fibrosis. However, the effects of CCN3, or CCN3 derivatives, on dermal fibrosis have yet to be studied. In this report, I aim to fill these gaps in knowledge. Using the bleomycin-induced model of SSc dermal fibrosis, I will investigate the anti-fibrotic potential of the CCN3-based peptide BLR-200.

3.2 Methods

3.2.1 Bleomycin-Induced Model of Dermal Fibrosis

Bleomycin sulfate (0.1 units/100 ml per injection; Sigma) or vehicle (PBS, 100 ml per injection) was injected subcutaneously into a single location on the flank of wild-type C57BL/6J (Jackson Laboratories) mice once daily for 28 days. Bleomycin-treated mice were further divided into two treatment groups, which were injected intraperitoneally 3

times per week with either scrambled peptide (10 µg/kg) or BLR-200 (10 µg/kg). At the end of the treatment period, mice were sacrificed via CO_2 inhalation, and skin and RNA samples were collected for analysis. All animal protocols were approved by the Animal Care and Veterinary Services at Western University.

3.2.2 Histological Assessment of Skin Thickness

Skin samples were fixed in a 4% paraformaldehyde (Sigma) solution overnight at 4°C, and were subsequently processed, and embedded in paraffin wax. The embedded samples were then sectioned (5µm), using a Leica microtome, and collected on Superfrost Plus slides (Thermo Fisher). Skin sections were deparaffinized using Xylenes (Sigma) and rehydrated by successive immersion in descending concentrations of alcohol. Sections were then stained with haematoxylin (Sigma) for 1 minute, rinsed in water, and counterstained with eosin-y (Thermo-Fisher) for 7 minutes. Sections were visualized on a Zeiss Imager M2m microscope (Carl Zeiss, Jena, Germany). For each section, 4-6 images were taken at random. Two different depths were analyzed for each mouse. Dermal thickness of the sections was measured using Northern Eclipse software. Measurements were analyzed by one-way ANOVA with Tukey's post hoc test ($p<0.05$).

3.2.3 Histological Collagen Analysis

Skin samples were fixed, processed, embedded, and sectioned as described above. Skin sections were deparaffinized using Xylenes (Sigma) and rehydrated by successive immersion in descending concentrations of alcohol. The sections were then subjected to Masson's trichrome stain, as previously described (Quesnel et al., 2019). Sections were visualized on a Zeiss Imager M2m microscope. For each section, 4-6 images were taken at random. Collagen deposition and density were analyzed using ImageJ by measuring the percent area of the dermis stained with alanine blue. Measurements were analyzed by one-way ANOVA with Tukey's post-hoc test ($p<0.05$).

Skin sections were also stained with picrosirius red to further analyze collagen content. Briefly, skin sections were subjected to incubation in a solution of 0.1% Sirius red (Sigma) in saturated picric acid (Thermo Fisher) for 60 minutes, followed by two washes with 0.5% acetic acid (Sigma). Sections were visualized under circularly polarized light to assess

collagen fibrillar hue through collagen birefringence properties. Images were taken at a constant light intensity, a fixed 45° angle to the polarizing filter, and the same analyzer was used to facilitate consistent comparisons. For each section, 4-6 images were taken at random. Collagen viewed using this method will appear different colours depending on fiber thickness and spatial orientation, with the colour changing from blue to yellow to orange to red as fiber density increases. These properties were used to determine the proportion of different collagen colours within the dermis, a method which has been used to quantitatively analyze collagen content (Rich and Whittaker, 2005). The relative colour content of the images can be obtained using ImageJ to separate the digital images into their hue, saturation, and value components, as previously described (Armstrong, 2019). Using this method, the relative amount of red, orange, yellow, and blue pixels within a given range can be expressed as a proportion of the total number of pixels representing collagen. The measurements were analyzed using a one-way ANOVA with Tukey's post hoc test ($p<0.05$).

3.2.4 Immunohistochemistry

3.2.4.1 3,3′-Diaminobenzidine (DAB) Immunostaining

Skin samples were fixed, processed, embedded, and sectioned as described above. Skin sections were deparaffinized using Xylenes (Sigma) and rehydrated by successive immersion in descending concentrations of alcohol. Sections were then stained using the Vectastain ABC Kit (Vector Laboratories). In brief, skin sections were subjected to sodium-citrate antigen retrieval for 30 minutes at 98°C. Non-specific binding was blocked by incubating slides with diluted 2.5% normal goat serum. Sections were then incubated with primary antibodies against αSMA (1:400; Abcam, ab5694) and YAP/TAZ (1:200; Cell Signaling, D24E4). This was followed by incubation with biotinylated secondary antibody, and then Vectastain ABC Reagent (Avidin DH, Biotinylated Horseradish Peroxidase H). Primary antibody binding was then visualized by incubation with ImmPact DAB peroxidase substrate (Vector Laboratories) and sections were counterstained with hematoxylin. Sections were visualized on a Zeiss Imager M2m using Zen Pro software. For each section, 4-6 images were taken at random and the proportion of positively stained

cells per field of view were measured using ImageJ. Measurements were analyzed by one-way ANOVA with Tukey's post-hoc test ($p<0.05$).

3.2.4.2 Immunofluorescence

Skin samples were fixed, processed, embedded, and sectioned as described above. Skin sections were deparaffinized using Xylenes (Sigma) and rehydrated by successive immersion in descending concentrations of alcohol. The sections were then subjected to sodium-citrate antigen retrieval for 30 minutes at 98°C, followed by blocking in a solution of 10% normal serum, 0.1% Triton X-100 (Sigma) and PBS for 1 hour. Slides were then incubated with primary antibody diluted in blocking solution overnight at 4°C. The primary antibodies used were: anti-CCN1 (1:200; Millipore, ABC102), anti-CCN2 (1:100; Santa Cruz, Sc-365970), anti-CCN3 (1:1000, antibody used as described by Kyurkchiev et al., 2004), and anti-Sox2 (1:200; Santa Cruz, Sc-365823). After incubation with primary antibody, sections were rinsed and incubated with the appropriate secondary antibody conjugated to Alexa Fluor 647 (Thermo Fisher) for 1 hour. Sections were then counterstained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher) and imaged on a Zeiss fluorescence microscope using Northern Eclipse software. For each section, 4-6 images were taken at random and the amount of positively stained cells in the dermis were assessed.

3.2.5 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

To collect RNA for qPCR analysis, skin tissue samples were first subjected to bead homogenization using a BeadBug homogenizer (Sigma). RNA was then purified from the samples using TRIzol reagent (Thermo Fisher) to solubilize biological material, followed by phenol-chloroform phase separation. The aqueous phase containing RNA was collected from the samples, and RNA was precipitated using isopropanol and collected by centrifugation at 12,000 rpm for 15 minutes at 4°C. The RNA pellet was then washed three times using 90% ethanol before resuspension in RNase-free water (Qiagen). RNA concentration and integrity were measured via Nanodrop and 1 µg of RNA was reverse transcribed using qScript cDNA SuperMix (Quantabio), producing a cDNA library. SYBR green real-time PCR was then performed by combining cDNA (7ng/well), SYBR master

mix (Thermo Fisher) and gene specific primers. Signal changes were detected using a ViiA7 Real-Time PCR System. The following gene-specific primers used: *Acta2*, *Ccn1*, *Ccn2, Ccn3, Itga11, Sox2, Yap1, Plod2,* and *Lox*. Samples were run in triplicate and expression values were standardized to control values from *Rn18s* primers using the ΔΔCt method. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test ($p<0.05$).

Target	**Forward** (5' to 3')	**Reverse** (5' to 3')
Rn18s	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
Acta2	CATCCGACACTGCTGACA	AGGTCTCAAACATAATCTGGGTCA
Ccn1	TCTGCGCTAAACAACTCAACGA	GCAGATCCCTTTCAGAGCGG
Ccn2	TGCTGTGCATCCTCCTACCG	CAGAGAGCGAGGAGCACCAA
Ccn3	GCGGGGAGAGTTGTTCTGAG	GTCTCCCTCTGGAACCATGC
Itga11	CGCTGTGAATCAGACGAGGT	CCCACAGGGCTATTCTTGT
Sox2	CTCCGCAGCGAAACGACAG	AGTCGGCATCACGGTTTTTG
Yap1	GAACTCGGCTTCAGGTCCTC	AGGGTCAAGCCTTGGGTCTA
Plod2	CAGGAACATGGGCATGGATTTC	GACGTGTCACAAGAGGAGCAA
Lox	AAATGGGTATCCAACAAATGGC	GTGCGTTAGAGGACAACAGGA

Table 3-1. Primers used for qPCR.

3.2.6 Proteomic Analysis

Proteomic analysis was carried out in collaboration with colleagues from the Proteomic Resource Facility (PRF) in the Department of Pathology at the University of Michigan, where mass spectrometry-based Tandem Mass Tag (TMT, Thermo Fisher) was employed. Full skin protein samples were homogenized and digested with trypsin. Protein fragments were individually labeled with one of ten isobaric mass tags following the manufacturer's protocol. After labelling, equal amounts of peptide from each condition were mixed. The labelled proteins were then fractionated by 2D-liquid chromatography, using basic pH reverse-phase separation followed by acidic pH reverse-phase. The samples were analyzed on a high-resolution, tribrid mass spectrometer (Orbitrap Fusion Tribrid, Thermo Fisher) using conditions optimized by the PRF. MultiNotch MS3 approach was employed to obtain accurate quantitation of the identified proteins. Data analysis was performed using Proteome Discoverer (v 2.3, Thermo Fisher). MS2 spectra were searched against the SwissProt reviewed mouse protein database (downloaded on 2019-06-29) using the following search parameters: MS1 and MS2 tolerance were set to 10 ppm and 0.6 Da, respectively; carbamidomethylation of cysteines (57.02146 Da) and TMT labeling of lysine and N-termini of peptides (229.16293 Da) were considered static modifications; oxidation of methionine (15.9949 Da) and deamidation of asparagine and glutamine (0.98401 Da) were considered variable. Identified proteins and peptides were filtered to retain only those that passed ≤2% false-discovery rate (FDR) threshold of detection. Quantitation was performed using reporter ion intensity extracted from high-quality MS3 spectra within a ±10 PPM window centered on the theoretical m/z value of each reporter ion. Reporter ion intensities were corrected for isotopic impurities of different TMT reagents as specified by the manufacturer. Only those peptide reporter ion intensities with an average signal-to-noise ratio of 9 and <40% co-isolation interference were considered for quantification. Differential protein expression between conditions, normalizing to control (PBS) for each subject's specimens separately was established using edgeR (Robinson et al., 2010). Then, results for individual proteins from six mice per treatment were pooled. Fold change ratios were produced for either bleomycin + scrambled mice or bleomycin + BLR-200 mice, relative to PBS. Differentially expressed proteins were

filtered based on a ±1.8-fold cut-off ($p<0.05$). Reactome V69 (reactome.org) was used for pathway enrichment analyses.

3.3 Results

3.3.1 BLR-200 prevents bleomycin-induced changes in skin thickness and collagen organization

To begin to assess the effect of BLR-200 on bleomycin-induced dermal fibrosis, skin sections were histologically examined using H&E and trichrome staining. Skin thickness and collagen deposition are commonly the most observable fibrotic changes that occur in bleomycin-induced dermal fibrosis (Liu et al., 2011; Quesnel et al., 2019). In the scrambled peptide control group (BLM; bleomycin), bleomycin injections caused an increase in dermal thickness; a change that was impaired in mice receiving BLR-200 treatment (BLR-200) (Figure 3-1). BLM mice also had increased collagen deposition in the dermis and exhibited consistently disorganized collagen organization (Figure 3-2A). These changes in collagen deposition and organization were diminished in BLR-200 mice.

To further assess fibrotic changes in collagen, qPCR analysis was employed to assess the mRNA expression of *Plod2* and *Lox*, genes that encode proteins that promote collagen crosslinking. PLOD2 hydroxylates lysine residues of collagen telopeptides, thus increasing the amount of pyridinoline cross-links, which promote irreversible accumulation of collagen in fibrotic tissue (Gjaltema et al., 2015; van der Slot et al., 2003). LOX facilitates the accumulation of stable collagen by increasing the amount of covalent cross-linking in collagen strands (Chen et al., 2018). Both PLOD2 and LOX protein expression are increased in fibroblasts isolated from lesional areas of SSc patients (Meyringer et al., 2007; Nguyen et al., 2021; van der Slot et al., 2003). In this study, BLR-200 treatment prevented the bleomycin-induced increase in *Plod2* and *Lox* mRNA expression (Figure 3-2B).

Finally, picrosirius red staining was used, in combination with circularly polarized light, as a highly sensitive means to visualize collagen fibers. Fibrillar hue was used to assess structural changes in collagen in mice treated with bleomycin +/- scrambled peptide (BLM) or BLR-200 (BLR-200). BLM mice contained a significantly higher proportion of yellow/orange-stained collagen, representing newly synthesized collagen (Figure 3-2C)

(Armstrong, 2019). BLR-200 impaired this bleomycin-induced increase in newly synthesized collagen.

Overall, these results suggest that BLR-200 treatment impairs general fibrotic changes associated with bleomycin-induced dermal fibrosis.

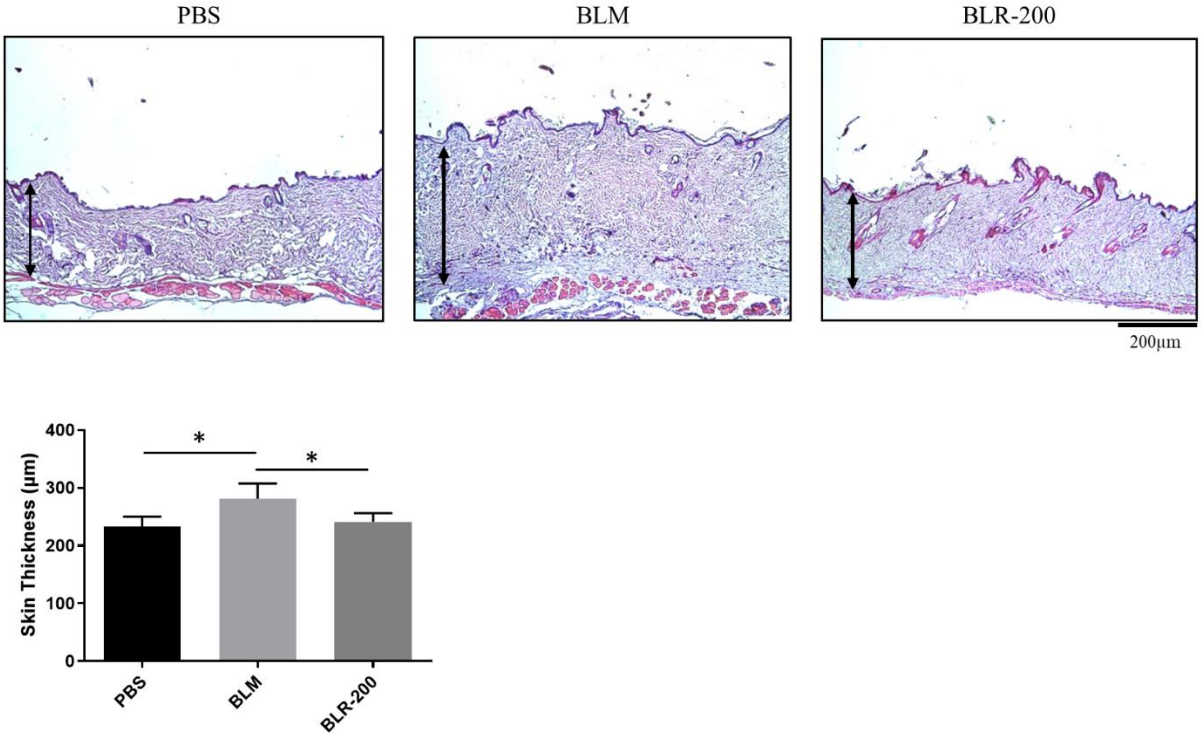
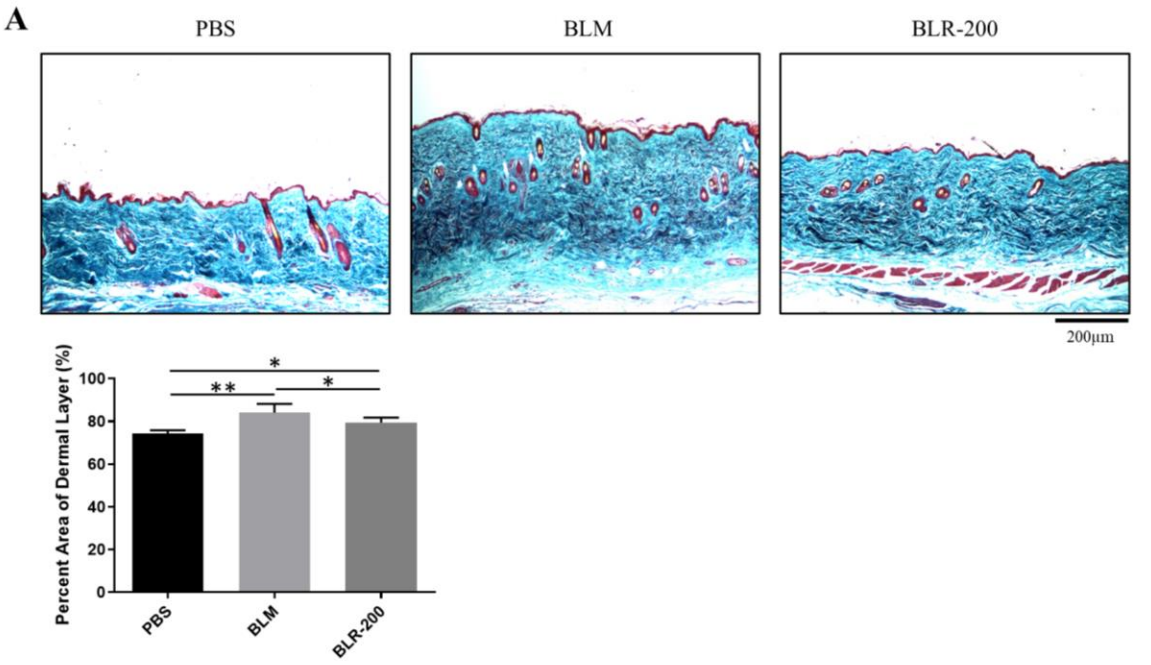
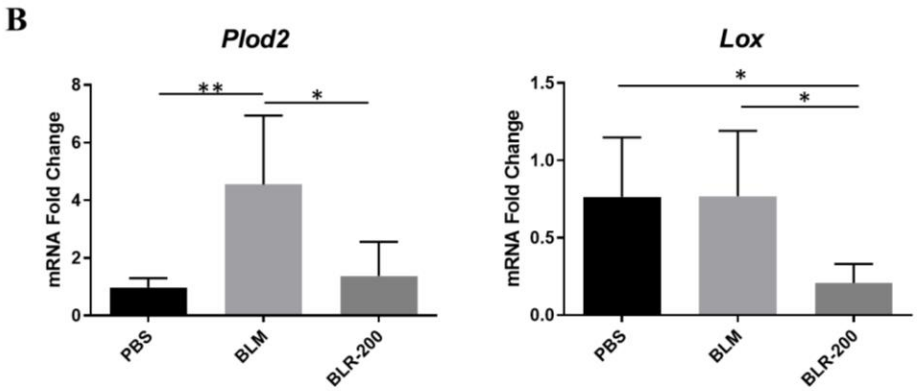


Figure 3-1. BLR-200 prevents the bleomycin-induced increase in skin thickness.

Bleomycin sulfate (0.1 units/100 ml per injection) or PBS (100 ml per injection) was injected subcutaneously into a single location on the flank of wild-type C57BL/6J mice once daily for 28 days. Bleomycin-treated mice were further divided into two treatment groups, which were injected intraperitoneally 3 times per week with either 10 µg/kg scrambled peptide (BLM) or 10 µg/kg BLR-200 (BLR-200). At the conclusion of the experiment, dermal tissue was fixed and stained with H&E. Representative images are shown. Dermal thickness measurements were taken at 3 different points along 2 depths of skin, averaged for each mouse, and compared among treatment groups (one-way ANOVA with Tukey's post hoc test; *$p<0.005$).


A
PBS
BLM
BLR-200
200μm
Percent Area of Dermal Layer (%)
100
80
60
40
20
0
*
**
*
PBS
BLM
BLR-200


B
Plod2
mRNA Fold Change
8
6
4
2
0
**
*
PBS
BLM
BLR-200
Lox
mRNA Fold Change
1.5
1.0
0.5
0.0
*
*
PBS
BLM
BLR-200

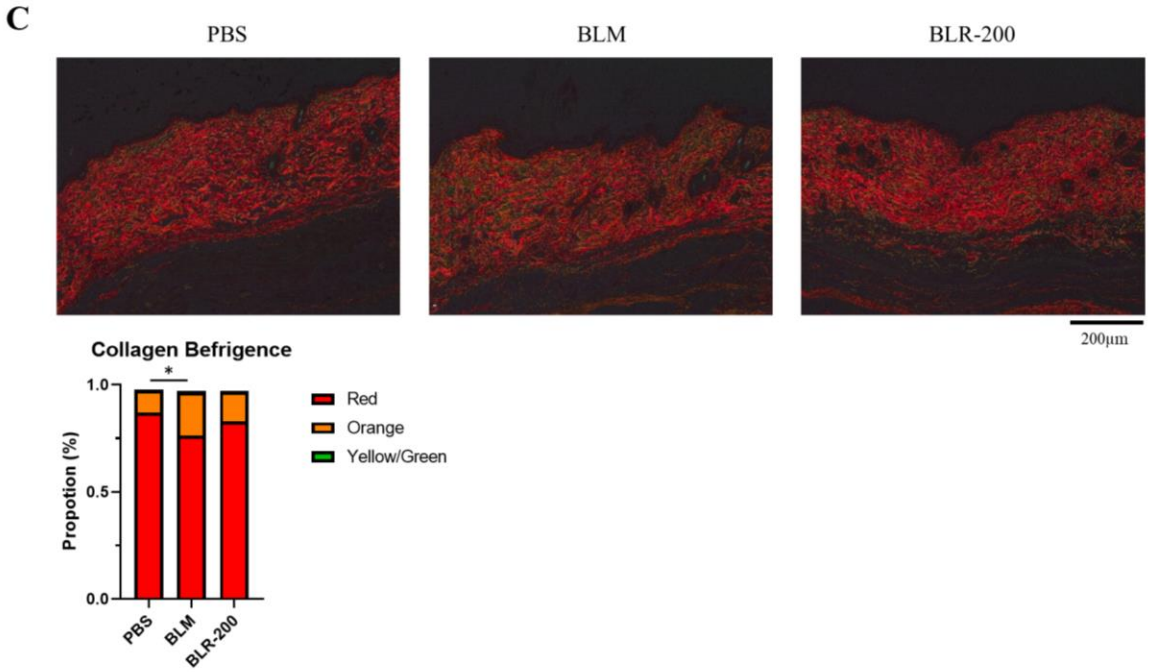
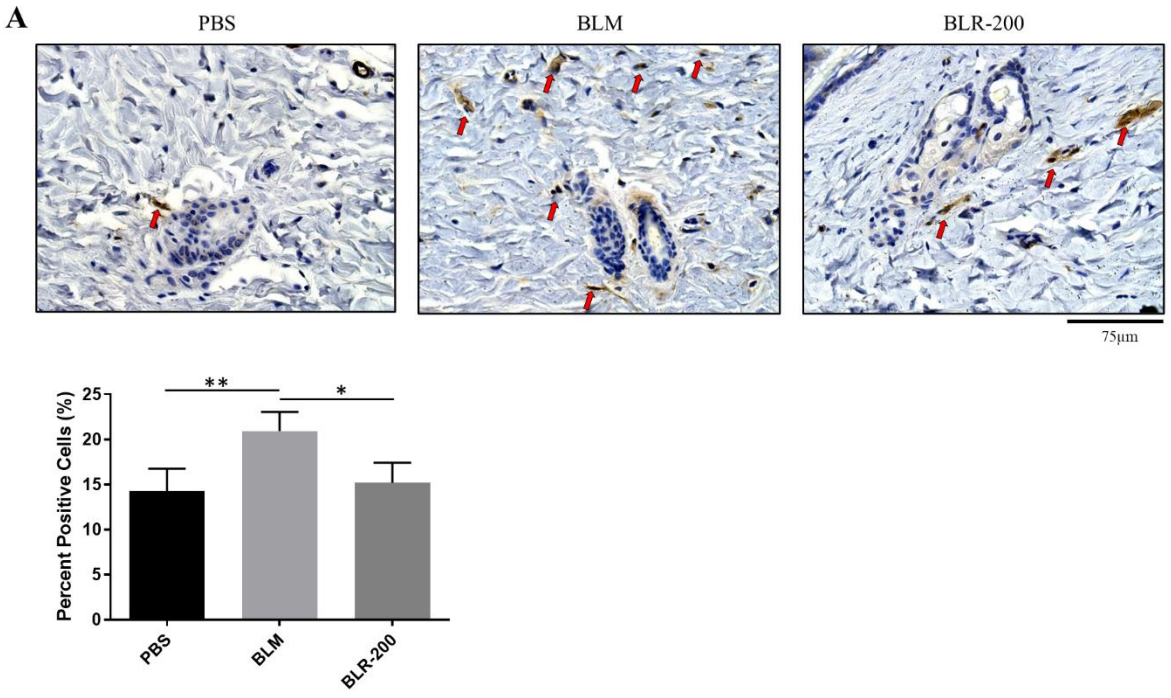

C
PBS
BLM
BLR-200
200μm
Collagen Befrigence
Propotion (%)
1.0
0.5
0.0
*
Red
Orange
Yellow/Green
PBS
BLM
BLR-200

Figure 3-2. BLR-200 prevents bleomycin-induced collagen changes in the dermis.

Wild-type C57BL/6J mice were either treated with PBS (n=6), bleomycin + scrambled peptide (BLM; n=6), or bleomycin + BLR-200 (BLR-200; n=6) **(A)** Dermal tissue was stained with Masson's trichrome stain to examine histological changes in collagen organization. Images were taken at 3 different points along 2 different depths of skin. Representative images are shown. The percent area of the dermal layer stained for trichrome was determined using ImageJ (one-way ANOVA with Tukey's post hoc test; **$p<0.002$; *$p<0.05$). **(B)** Total RNA was obtained from skin and subjected to SYBR Green real-time PCR. Primers were used to detect collagen cross-linking genes *Plod2* and *Lox*. Relative gene expression, compared to the housekeeping gene *Rn18s*, was determined using the ΔΔCt method (one-way ANOVA and Tukey's post-host test; **$p<0.002$; *$p<0.05$). **(C)** Skin sections were stained with picrosirius red and collagen birefringence properties were analyzed to investigate newly synthesized collagen and fiber density. A one-way ANOVA and Tukey's post-host test were used to analyze the data (*$p<0.05$).

3.3.2 BLR-200 treatment prevents the bleomycin-induced increase in αSMA-positive myofibroblasts

A hallmark of progressive fibrosis is the presence of activated myofibroblasts characterized by the expression of the highly contractile αSMA (Gabbiani, 2003). In order to assess the amount of myofibroblasts expressing αSMA, skin sections from the fibrotic lesion were examined using DAB immunostaining with an anti-αSMA antibody. Compared to PBS-injected control mice, mice subcutaneously injected with bleomycin showed, in the presence of scrambled peptide, a significant increase in the amount of αSMA-expressing myofibroblasts in the dermis. This increase in number of αSMA-expressing myofibroblasts in the presence of bleomycin was significantly decreased in the presence of BLR-200 (Figure 3-3A). Finally, RNA was extracted from whole skin and subjected to SYBR Green qPCR with primers detecting *Acta2* (murine gene encoding αSMA) and showed similar trends (Figure 3-3B). These results indicate that BLR-200 treatment is able to prevent activation of highly contractile myofibroblasts in the fibrotic lesion.


A
PBS
BLM
BLR-200
75µm
Percent Positive Cells (%)
25
20
15
10
5
0
**
*
PBS
BLM
BLR-200

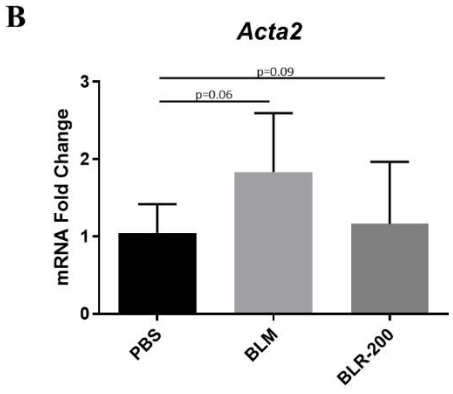

B
Acta2
mRNA Fold Change
3
2
1
0
p=0.09
p=0.06
PBS
BLM
BLR-200

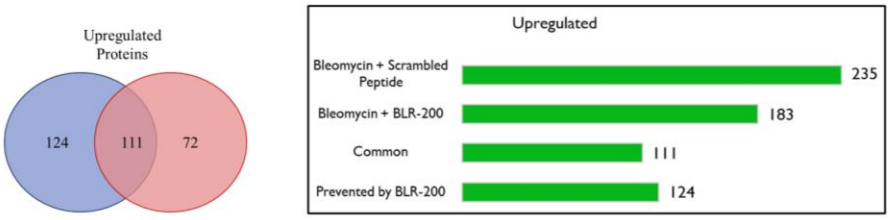
Figure 3-3. BLR-200 impairs bleomycin-induced increase in αSMA-expressing myofibroblasts.

Wildtype C57BL/6J mice were either treated with PBS (n=6), bleomycin + scrambled peptide (BLM; n=6), or bleomycin + BLR-200 (BLR-200; n=6). **(A)** Fixed dermal tissue sections were incubated with an anti-αSMA antibody and antibody localization was visualized using DAB chromogen. Representative images are shown. The percentage of fibroblasts positive for αSMA was determined using ImageJ (one-way ANOVA and Tukey's post hoc test; **$p<0.002$; *$p<0.05$). **(B)** Total RNA was extracted from the skin and subjected to SYBR Green real-time PCR using primers detecting *Acta2* (murine gene encoding αSMA). Relative *Acta2* expression, compared to the housekeeping gene *Rn18s*, was determined using the ΔΔCt method (one-way ANOVA and Tukey's post-hoc test; *$p<0.05$).

3.3.3 Proteomic analysis reveals that BLR-200 prevents the bleomycin-induced increase in ECM-related proteins

To further assess the effect of BLR-200 on bleomycin-induced dermal fibrosis, I employed an unbiased proteomic approach. Protein was extracted from full skin and subjected to tandem mass-tag mass spectrometry. Protein expression for BLM mice and BLR-200 mice was normalized to protein expression in the control PBS mice, producing fold change differences. A list of upregulated proteins in each experimental group was generated using a 1.8-fold cut-off ($p<0.05$). Out of the upregulated proteins in BLM mice, 124/235 were prevented by BLR-200 treatment (Figure 3-4A). Proteins that were induced in BLM mice, but not BLR-200 mice were analyzed using the Reactome pathway database. It was found that several ECM-related pathways were prevented by BLR-200 treatment, including extracellular matrix organization (R-MMU-1474244), assembly of collagen fibrils and other multimeric structures (R-MMU-2022090), laminin interactions (R-MMU-3000157) and collagen formation (R-MMU-1474290) (Figure 3-4B). Further analysis revealed that BLR-200 treatment specifically prevents induction of several ECM-associated proteins, including laminin (lam)a5, lama3, lamb3, lamc2, nectin-2, pecam-1, and fibromodulin (Figure 3-4C). Laminins, glycoproteins in the extracellular matrix, promote cell adhesion and migration; they may also play a role in fibroblast proliferation (Domogatskaya et al., 2012; Liu et al., 2020). Fibromodulin, a proteoglycan that plays a role in collagen fibril assembly (Kalamajski et al., 2016), has been linked to cardiac remodeling and liver fibrosis (Andenæs et al., 2018; Mormone et al., 2012). Nectin-2 and Pecam-1, cell adhesion molecules, enhance inflammation, cell adhesion, and cell migration (Woodfin et al., 2007; Yamada et al., 2017). Collectively, these results provide further evidence that BLR-200 prevents bleomycin-induced skin fibrosis.

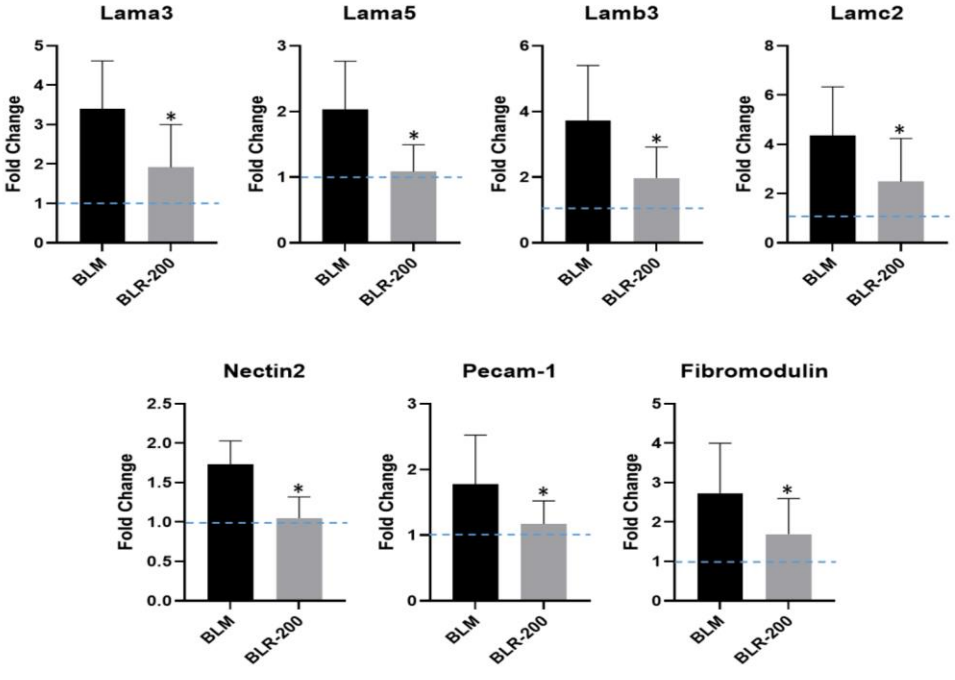
A



B

Pathway Identifier	Pathway name	Entities pValue
R-MMU-6805567	Keratinization	1.11E-16
R-MMU-6809371	Formation of the cornified envelope	1.11E-16
R-MMU-1266738	Developmental Biology	1.59E-09
R-MMU-446107	Type I hemidesmosome assembly	1.22E-08
R-MMU-2559584	Formation of Senescence-Associated Heterochromatin Foci	9.90E-08
R-MMU-1474244	Extracellular matrix organization	1.20E-05
R-MMU-140342	Apoptosis induced DNA fragmentation	1.20E-05
R-MMU-75153	Apoptotic execution phase	2.77E-05
R-MMU-2022090	Assembly of collagen fibrils and other multimeric structures	6.18E-05
R-MMU-6799990	Metal sequestration by antimicrobial proteins	1.33E-04
R-MMU-2559586	DNA Damage/Telomere Stress Induced Senescence	2.13E-04
R-MMU-446728	Cell junction organization	2.60E-04
R-MMU-3000157	Laminin interactions	6.12E-04
R-MMU-1474290	Collagen formation	6.49E-04

C



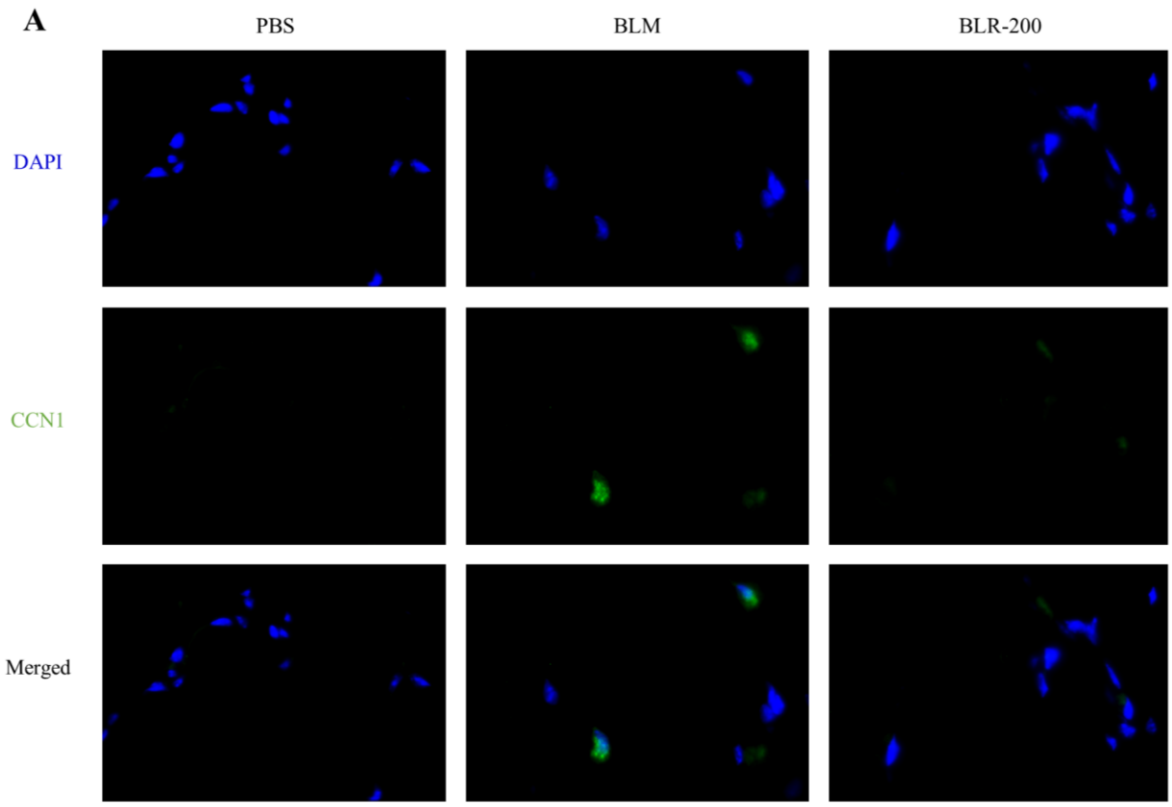
(----) = PBS baseline expression

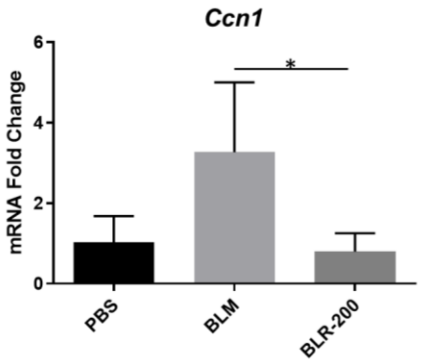
Figure 3-4. BLR-200 prevents changes in protein expression patterns associated with bleomycin-induced dermal fibrosis.

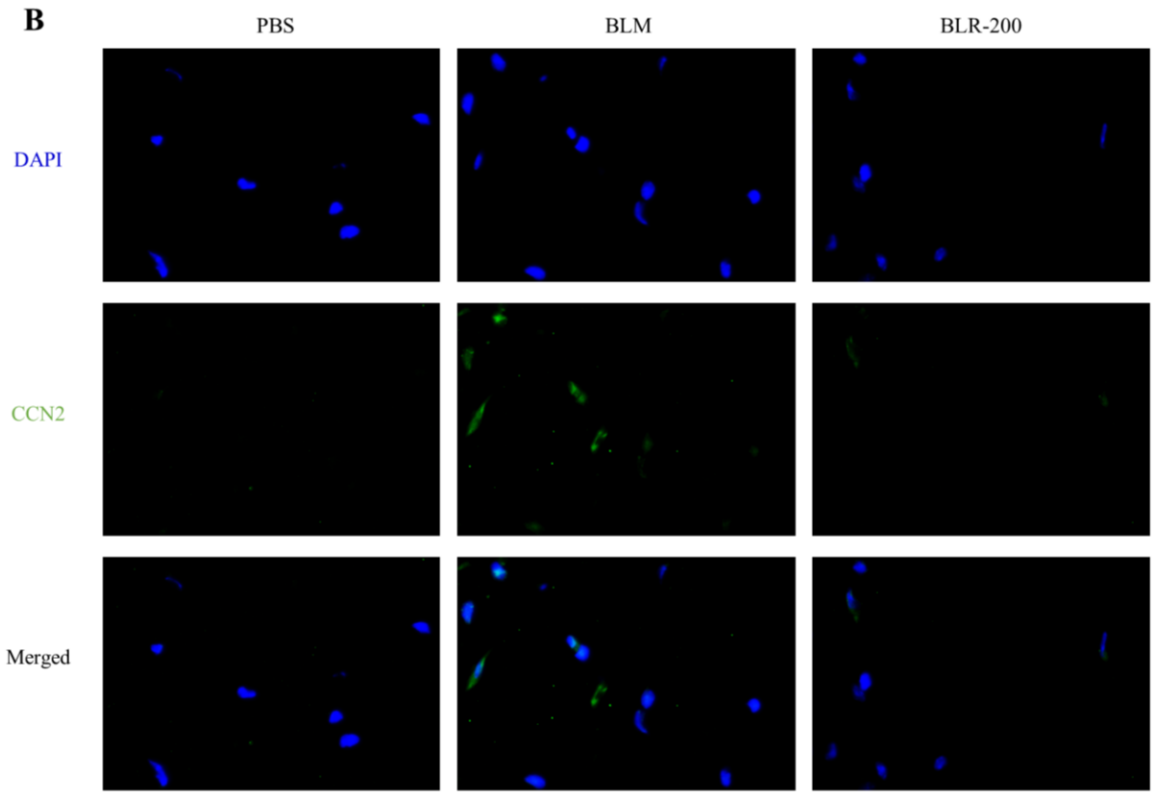
Wildtype C57BL/6J mice were either treated with PBS (n=6), bleomycin + scrambled peptide (BLM; n=6), or bleomycin + BLR-200 (BLR-200; n=6). Total protein was extracted from the skin and subjected to tandem mass-tag mass spectrometry. Relative protein expression for BLM mice and BLR-200 mice was normalized to the control PBS group, generating fold change differences for all detected proteins. A list of upregulated proteins in each experimental group was generated using a 1.8-fold cut-off ($p<0.05$). **(A)** Venn diagram showing that 124 proteins that were induced in BLM mice are prevented by BLR-200 treatment. **(B)** Reactome pathway analysis of bleomycin-induced proteins that were prevented by BLR-200 treatment shows that the many of the top pathways prevented include ECM-associated processes, including keratinization, extracellular matrix organization, collagen formation, and laminin interactions. **(C)** Examining fold change differences of individual proteins revealed that specific laminins (a3, a5, b3, c2) and fibrosis-related proteins (nectin-2, pecam-1, fibromodulin) were prevented by BLR-200 treatment. A student's t-test was used to analyze differences in fold change (*$p<0.05$).

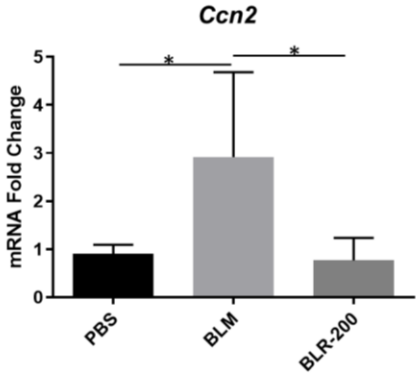
3.3.4 BLR-200 prevents the bleomycin-induced increase in CCN1 and CCN2 expression

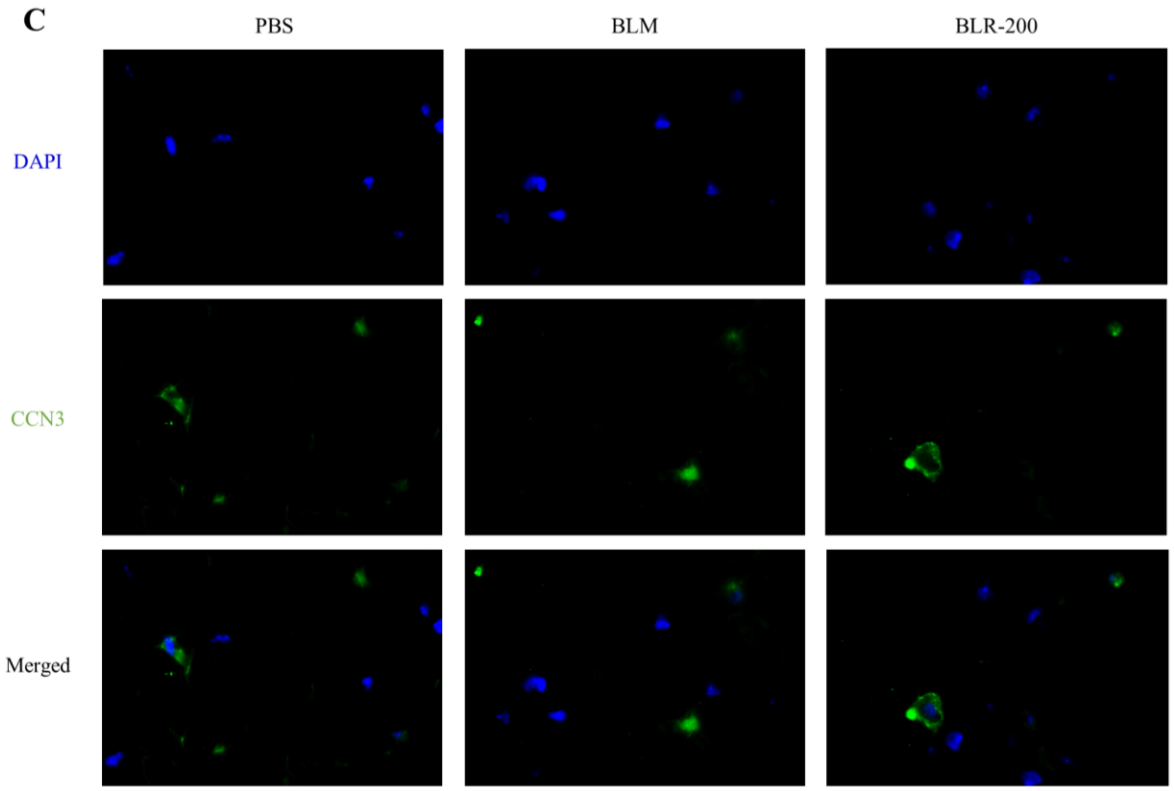
Both CCN1 and CCN2 are markers and mediators of bleomycin-induced skin fibrosis (Liu et al., 2011; Quesnel et al., 2019). Furthermore, I have shown that CCN1 and CCN2 are reciprocally regulated to CCN3 in response to TGFβ1 in dermal fibroblasts. Moreover, ample evidence suggests that CCN3 may have inhibitory activity on CCN1 and CCN2 (Riser et al., 2014). Based on these observations, I anticipated that the CCN3-based peptide, BLR-200, would suppress the bleomycin-induced increases in CCN1 and CCN2 protein and mRNA expression. To test this notion, I used immunohistochemistry, to detect protein localization within the dermis, and qPCR, to detect mRNA expression. As anticipated the detected protein levels of both CCN1 and CCN2 were significantly increased in the dermis of BLM mice; however, these increases were not observed in BLR-200 treated mice (Figure 3-5A and Figure 3-5B). Similar results were observed when gene expression of *Ccn1* and *Ccn2* was detected. Conversely, no significant alterations in CCN3 protein localization or *Ccn3* gene expression were observed with any treatment (Figure 3-5C).


A
PBS
BLM
BLR-200
DAPI
CCN1
Merged


Ccn1
mRNA Fold Change
6
4
2
0
*
PBS
BLM
BLR-200


B
PBS
BLM
BLR-200
DAPI
CCN2
Merged


Ccn2
mRNA Fold Change
0
1
2
3
4
5
*
*
PBS
BLM
BLR-200


C
PBS
BLM
BLR-200
DAPI
CCN3
Merged

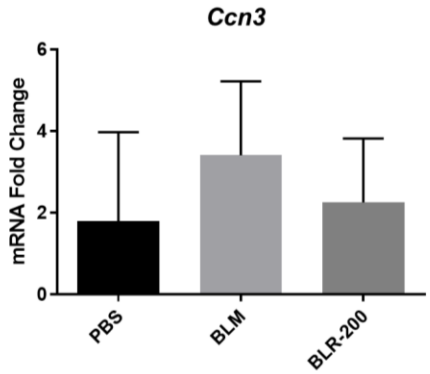
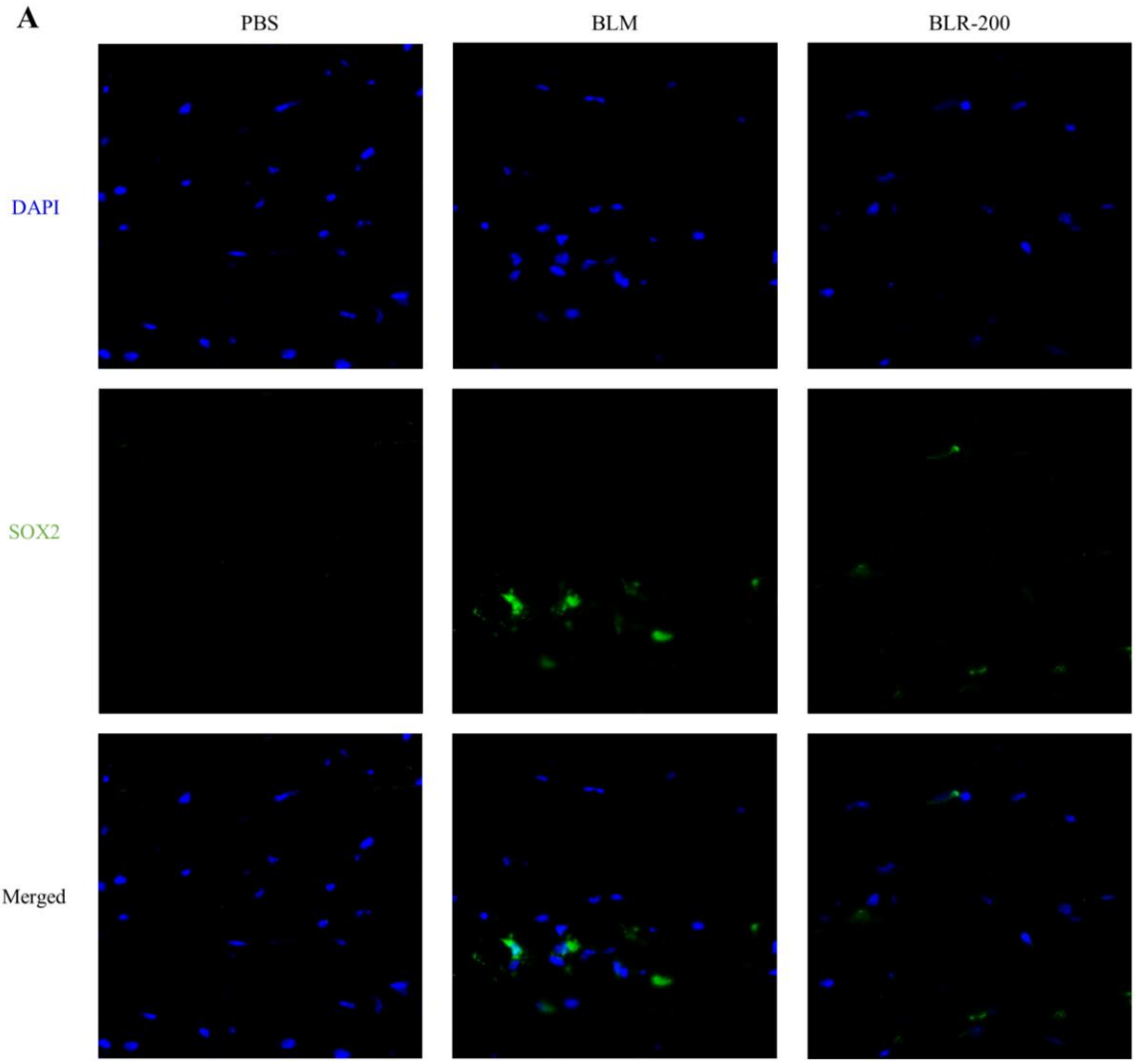

Ccn3
mRNA Fold Change
6
4
2
0
PBS
BLM
BLR-200

Figure 3-5. BLR-200 treatment prevents bleomycin-induced changes in CCN1 and CCN2.

Wildtype C57BL/6J mice were either treated with PBS (n=6), bleomycin + scrambled peptide (BLM; n=6), or bleomycin + BLR-200 (BLR-200; n=6). Dermal tissue was fixed, stained and visualized by immunohistochemistry with primary antibodies against CCN1, CCN2, and CCN3. Representative images are shown. Total RNA was also extracted from the skin and subjected to SYBR Green real-time PCR using primers detecting *Ccn1*, *Ccn2* and *Ccn3*. Relative mRNA expression, compared to the housekeeping gene *Rn18s*, was determined using the ΔΔCt method (one-way ANOVA and Tukey's post-hoc test; *$p<0.05$). BLR-200 prevents bleomycin-induced increase in **(A)** CCN1 and **(B)** CCN2 protein and mRNA expression. **(C)** No significant changes were observed with CCN3 expression.

3.3.5 BLR-200 prevents the bleomycin-induced increase of the plasticity marker SOX2 in the reticular dermis

The origin of pro-fibrotic αSMA-expressing myofibroblasts is unclear, although emerging evidence suggests these cells are derived from plastic dermal fibroblasts via a progenitor cell-like intermediate (Chadli et al., 2019; Tsang et al., 2019). In bleomycin-induced skin fibrosis, a majority of myofibroblasts stain positive for the progenitor/plasticity marker SOX2 (Liu et al., 2014). Furthermore, CCN2 expression by SOX2-expressing cells has been shown to contribute to recruitment of these progenitor cells to the fibrotic lesion in bleomycin-induced fibrosis (Tsang & Leask, 2014). I therefore wanted to test the hypothesis that BLR-200 could reduce the appearance/recruitment of SOX2-positive cells to the dermis. To test this notion, skin sections were stained with an anti-SOX2 antibody. SOX2-positive cells were significantly increased in the reticular dermis of BLM mice, but not in BLR-200 mice (Figure 3-6A). In models of melanoma metastasis, a subset of αSMA-expressing myofibroblasts (myoCAFs) express high levels of both SOX2 and α11 integrin; this subtype of myoCAFs negatively correlates with disease-free survival (Tsang et al., 2019; Zeltz et al., 2019). Moreover, α11 integrin expression contributes to myofibroblast differentiation in multiple fibroblast types *in vitro* (Talior-Volodarsky et al., 2012; Tsang et al., 2019). Accordingly, whole skin tissue samples were also subjected to qPCR to detect *Sox2* and *Itga11* mRNA expression. I found that both *Sox2* and *Itga11* expression were increased in BLM mice; these increases were prevented by BLR-200 treatment (Figure 3-6B).


A
PBS
BLM
BLR-200
DAPI
SOX2
Merged

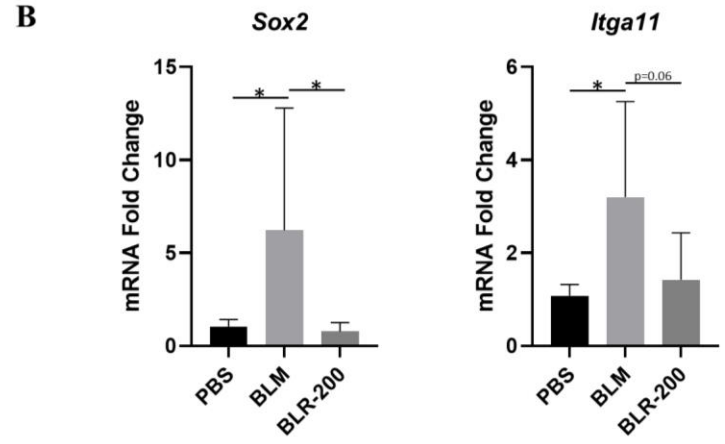

B
Sox2
Itga11
mRNA Fold Change
15
10
5
0
6
4
2
0
*
*
p=0.06
PBS
BLM
BLR-200

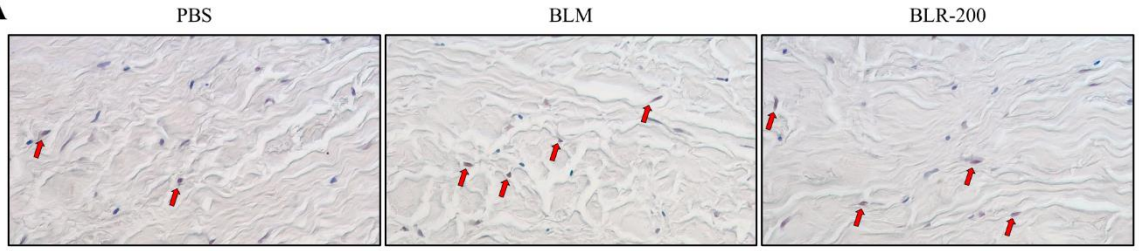
Figure 3-6. BLR-200 treatment prevents expression of the plasticity marker SOX2.

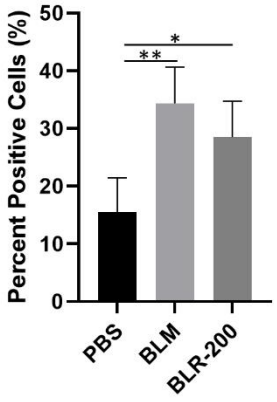
Wildtype C57BL/6J mice were either treated with PBS (n=6), bleomycin + scrambled peptide (BLM; n=6), or bleomycin + BLR-200 (BLR-200; n=6). **(A)** Dermal tissue was fixed, stained and visualized by immunohistochemistry with primary antibodies against SOX2. Representative images are shown. **(B)** Total RNA was extracted from the skin and subjected to real-time PCR using primers detecting *Sox2* and *ItgA11*. Relative *Sox2* and *Itga11* expression, compared to the housekeeping gene *Rn18s*, was determined using the ΔΔCt method (one-way ANOVA and Tukey's post-hoc test; *$p<0.05$).

3.3.6 The anti-fibrotic activity of BLR-200 is likely downstream of YAP1 mechanotransduction signaling

Upregulation and activation of the mechanosensitive transcriptional cofactor YAP1 promotes the perpetuation of pro-contractile myofibroblasts that are essential for fibrosis (Schulz et al., 2018; Shi-wen et al., 2021). Constitutive nuclear localization of activated YAP1 is also a feature of fibroblasts derived from the fibrotic lesions of SSc patients (Toyama et al., 2018). Since BLR-200 treatment is able to prevent multiple fibrotic changes in bleomycin-induced fibrosis, I wanted to assess the expression of YAP1 in the dermis of experimental mice. To assess the number of cells expressing YAP1, skin sections were examined using DAB immunostaining with an anti-YAP1 antibody. Compared to PBS-injected mice, BLM mice showed, in the presence of scrambled peptide, a significant increase in the number of YAP1-expressing cells in the dermis. Unexpectedly, the number of YAP1-expressing cells was not significantly altered by BLR-200 treatment (Figure 3-7A). Whole skin sections were also subjected to SYBR Green qPCR analysis with primers for *Yap1* and showed no significant changes (Figure 3-7B). Although the nuclear localization of YAP1 was not assessed, these results indicate that BLR-200 may be exhibiting its anti-fibrotic effects downstream of YAP mechanotransduction signaling.

A


PBS
BLM
BLR-200


Percent Positive Cells (%)
50
40
30
20
10
0
*
**
PBS
BLM
BLR-200

B

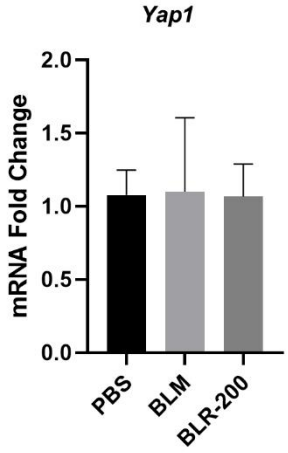

Yap1
mRNA Fold Change
2.0
1.5
1.0
0.5
0.0
PBS
BLM
BLR-200

Figure 3-7. BLR-200 treatment does not significantly effect bleomycin-induced changes in YAP1 expression in the dermal layer.

Wildtype C57BL/6J mice were either treated with PBS (n=6), bleomycin + scrambled peptide (BLM; n=6), or bleomycin + BLR-200 (BLR-200; n=6). **(A)** Fixed dermal tissue sections were incubated with an anti-YAP1 antibody and antibody localization was visualized using DAB chromogen. Representative images are shown. The percentage of fibroblasts positive for YAP1 was determined using ImageJ (one-way ANOVA with Tukey's post hoc test; *$p<0.002$; *$p<0.05$). **(B)** Total RNA was extracted from the skin and subjected to SYBR Green real-time PCR using a primer for *Yap1*. Relative *Yap1* expression, compared to the housekeeping gene *Rn18s*, was determined using the ΔΔCt method (one-way ANOVA and Tukey's post-hoc test; *$p<0.05$).

3.4 Discussion

Currently, only limited treatment options exist for SSc patients, highlighting the need to identify novel anti-fibrotic therapies. In recent years, mechanisms underlying fibrogenesis, including in SSc, have been the target of many studies. The CCN family of matricellular proteins has emerged as an important player in the development of skin and organ fibrosis in SSc. Prominent members of this family, CCN1 and CCN2 are upregulated in the connective tissue of early onset SSc patients and likely have pro-fibrotic effects (Dziadzio et al., 2005; Gardner et al., 2006; Sonnylal et al., 2010). In contrast, CCN3 is downregulated in SSc (Xu Shiwen and Richard Stratton, unpublished data), and has anti-fibrotic effects in multiple models of fibrosis, including the kidney (Riser et al., 2014). In this report, I show that targeting the regulation and activity of CCN proteins using BLR-200, a proprietary 14 amino acid peptide corresponding to an amino acid sequence within CCN3, prevents fibrosis in the bleomycin-induced model of SSc.

Mice injected subcutaneously with bleomycin exhibit vasculopathy, leading to inflammatory changes that recapitulate those which occur in the early stages of SSc (Yamamoto et al., 1999; Yamamoto & Katayama, 2011). Localized inflammation permits a fibrotic ECM, leading to activation of myofibroblasts and deposition of collagen, culminating in skin thickening due to fibrosis (King et al., 2018). A hallmark of fibrotic disease is the persistent presence of activated contractile myofibroblasts characterized by expression of αSMA (Darby et al., 1990). In this study, BLR-200 prevented the bleomycin-induced increase in skin thickness, collagen deposition and αSMA-expressing activated myofibroblasts. These are classical markers of bleomycin-induced dermal fibrosis and these data indicate that BLR-200 can prevent generalized fibrotic changes in this model. These results closely align with previous studies showing that loss of CCN2 by fibroblasts prevents bleomycin-induced fibrosis (Liu et al., 2011), supporting the idea that BLR-200 has effects mirroring CCN2 deficiency. My data also show that BLR-200 prevented the bleomycin-induced changes in collagen synthesis, organization, and mRNA expression of collagen cross-linking genes *Plod2* and *Lox*. PLOD2 is upregulated in fibrotic conditions, including in SSc, and promotes irreversible accumulation of collagen (Nguyen et al., 2021; van der Slot et al., 2003). LOX also facilitates over-accumulation of stable collagen in

fibrotic conditions (Chen et al., 2018). Stable, irreversible collagen deposition is a hallmark of fibrotic disorders and contributes to the mechanical tension and subsequent myofibroblast activation within the fibrotic microenvironment. Thus, prevention of *Plod2* and *Lox* expression is an important finding in this study. Previous studies have also shown that CCN1 plays a pro-fibrotic role in facilitating collagen synthesis and accumulation in the skin, and loss of CCN1 has direct effects on mRNA expression of *Plod2* and *Lox* (Quesnel et al., 2019). Collectively, these data indicate that the anti-fibrotic activity of BLR-200 operates through preventing the pro-fibrotic activity of both CCN1 and CCN2.

Providing further support of this idea, my data also indicate that BLR-200 treatment prevents the bleomycin-induced increase in *Ccn1* and *Ccn2* mRNA expression. Moreover, the number of cells expressing either CCN1 or CCN2 in the dermis was prevented by BLR-200 treatment. As previously mentioned, CCN3 has been proposed to be an endogenous inhibitor of CCN2 and has opposing effects to both CCN1 and CCN2 in models of diabetic nephropathy (Riser et al., 2009). Until now, the ability of CCN3 to prevent CCN1 and CCN2 expression in animal models of dermal fibrosis has not been studied. Thus, my findings provide valuable insights on the anti-fibrotic activity of CCN3. It is not known whether CCN3 has a direct inhibitory effect on the regulation of these proteins, or if this inhibition is downstream of BLR-200's effects. Further studies will be required to determine the exact mechanism of BLR-200 activity.

Unbiased proteomic analysis revealed that BLR-200 prevented the bleomycin-induced increase in expression of ECM-related proteins. In my analysis, I found that bleomycin-induced fibrosis caused an increase in proteins related to ECM organization and collagen formation, including nectin-2, pecam-1, fibromodulin, and several laminins (a5, a3, b4, and c3). Treatment with BLR-200 attenuated these changes. Fibromodulin is upregulated in multiple models of fibrosis and plays a role in collagen fibril assembly, having been shown to directly interact and enhance the activity of LOX (Andenæs et al., 2018; Kalamajski et al., 2016; Mormone et al., 2012). Thus, BLR-200 treatment seems to be affecting multiple facets of bleomycin-induced collagen deposition and organization. Laminins promote cell adhesion, differentiation, migration, and proliferation (Domogatskaya et al., 2012). They are upregulated in multiple fibrotic conditions including liver fibrosis and in the serum of

patients with SSc (Kanaizuka et al., 1991; Santos et al., 2005). They have also been shown to facilitate the TGFβ-induced expression matrix degrading enzymes in synovial fibroblasts (Hoberg et al., 2007). Moreover, Lama5 has been shown to modulate fibroblast proliferation and adhesive signaling in epidural fibrosis (Liu et al., 2020). Collectively, these data further emphasize the ability of BLR-200 to prevent fibrosis in the bleomycin model of skin fibrosis.

Previous experiments examining the origin of myofibroblasts in the fibrotic lesions of bleomycin-induced dermal fibrosis have revealed that plastic dermal fibroblasts become induced to express the progenitor cell marker SOX2, and these cells become activated into an αSMA-expressing myofibroblast, in a CCN2-dependent manner (Liu et al., 2014; Tsang & Leask, 2014). BLR-200 treatment prevented the bleomycin-induced overexpression of *Sox2* mRNA and reduced the number of SOX2-positive cells in the reticular dermis. Taken together with the finding that BLR-200 prevented the number of αSMA-expressing myofibroblasts, my data indicate that BLR-200 treatment likely impairs the CCN2-dependent activation of plastic dermal fibroblasts into myofibroblasts.

Recent evidence suggests that the persistent activated myofibroblast phenotype in fibrotic lesions occurs due to a constitutively activated, pro-adhesive and mechanotransducive autocrine signaling loop. One of the key mediators of mechanotransduction in myofibroblasts is the transcriptional cofactor YAP1. YAP1 is activated downstream of adhesive signaling and mediates the transcription of several pro-fibrotic and pro-adhesive factors, including αSMA and CCN2 (Dupont et al., 2011; Leask et al., 2003; Shi-wen et al., 2021). Increasing expression of these genes contributes to a pro-fibrotic microenvironment that further stimulates myofibroblast activation. Since YAP1 is a known mediator of this response, I wanted to investigate the effects of BLR-200 on its expression. Unexpectedly, BLR-200 treatment did not appear to have any significant effects on the bleomycin-induced increase in expression of YAP1. Since BLR-200 prevents expression of downstream targets of YAP1, including *Ccn1* and *Ccn2*, I expected to see alterations in YAP1 expression. Thus, these data indicate that the anti-fibrotic effects of BLR-200 are likely downstream of YAP1 signaling. It is worth noting that I could not assess the nuclear localization of YAP1. Activated YAP1 translocates to the nucleus, and constitutive nuclear

localization of YAP1 is a feature of fibroblasts from SSc patients (Toyama et al., 2018). Therefore, it is not known whether the observed YAP1-positive cells were expressing activated YAP1. In future studies, this should be taken into consideration. Collectively, these results shed light on the highly specific anti-fibrotic effects of BLR-200 and highlight the need to elucidate the exact mechanism of action.

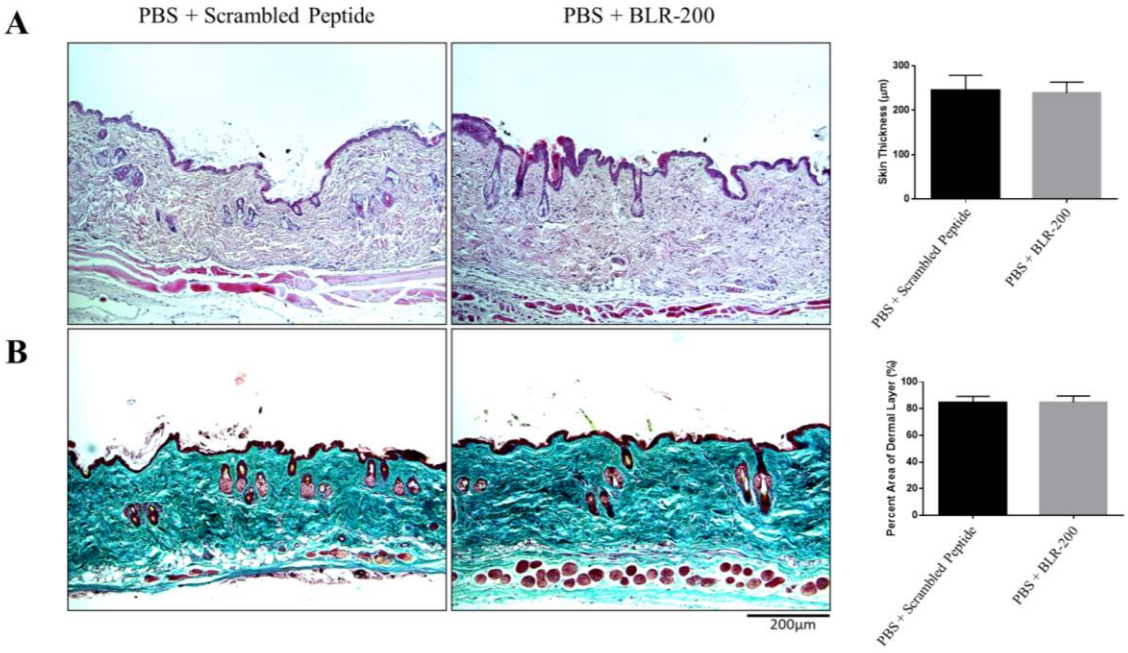
Overall, my results show remarkable consistency with previous studies aimed at targeting CCN2 using the monoclonal antibody FG-3019 in an Angiotensin II (Ang II)-induced model of dermal fibrosis. (Makino et al., 2017). In this study, FG-3019 treatment mitigated Ang II-induced fibrosis, as visualized by a reduction in skin thickness, collagen deposition, and the number of αSMA-expressing myofibroblasts. FG-3019 is currently in phase III clinical trials for IPF (Clinicaltrials.gov, NCT04419558), Duchene muscular dystrophy (Clinicaltrials.gov, NCT04632940), and pancreatic cancer (Clinicaltrials.gov, NCT03941093). Since BLR-200 and FG-3019 have similar anti-fibrotic effects in dermal fibrosis, this may reflect the ability to extend the therapeutic applications of BLR-200. Considering that antibody treatments often lack efficacy in fibrotic conditions due to difficulties with tissue penetration (Brenner et al., 2016; Epenetos et al., 1986; Piersma et al., 2020), BLR-200 may represent a more viable therapeutic approach.

The bleomycin-induced model is well-established in the study of SSc dermal fibrosis (Yamamoto, 2006). After 28 days, the fibrotic lesion of injected mice adequately recapitulates the fibrotic skin of SSc patients. For that reason, this model is ideal for investigating the overall fibrotic changes prevented by BLR-200 at this endpoint. However, at this point in the model, active inflammation is not usually occurring (Yamamoto & Katayama, 2011). Therefore, a limitation of this study is that the early pro-inflammatory stages of fibrogenesis cannot be assessed. To assess the effects of BLR-200 on active inflammation, future studies should focus on earlier timepoints in the model, for example after 10 days. A more comprehensive examination of BLR-200's effects at multiple timepoints would also provide valuable insights on the mechanism of action.

The mechanism of CCN protein activity, including their exact contribution to pathological conditions such as fibrosis, has remained elusive. Considering their highly context-

dependent effects, evaluation of their biological activity unquestionably requires the use of animal models. Cell culture models and *in vitro* assays are severely limited by their inability to recapitulate the specific components of a pathological microenvironment. Thus, to truly understand the biological significance of specific CCN proteins, for example the anti-fibrotic role of CCN3, animal models should be used. To date, very few studies have investigated the anti-fibrotic activity of CCN3 *in vivo*. For the first time, I have shown that a CCN3-based therapy impairs the establishment of fibrosis in a murine model of SSc dermal fibrosis, including prevention of CCN1 and CCN2 expression. This emphasizes the therapeutical potential of using CCN3-based peptides to treat fibrosis and highlights the importance of studying CCN proteins in animal models of disease.

3.5 Supplementary Figures


A
PBS + Scrambled Peptide
PBS + BLR-200
B
200μm
Skin Thickness (μm)
300
200
100
0
PBS + Scrambled Peptide
PBS + BLR-200
Percent Area of Dermal Layer (%)
100
80
60
40
20
0
PBS + Scrambled Peptide
PBS + BLR-200

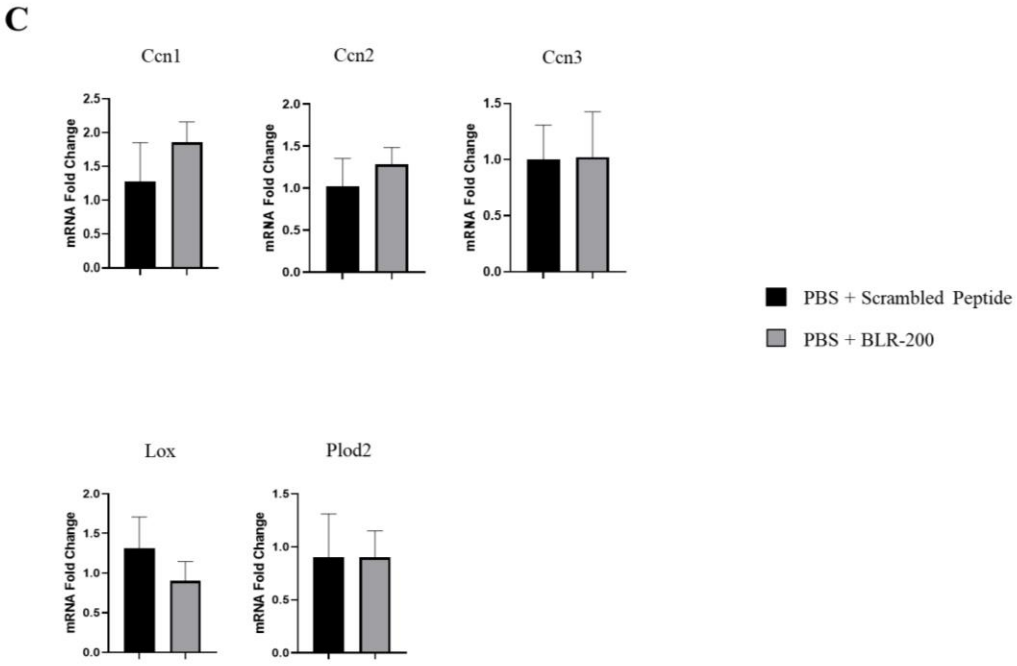

C
Ccn1
Ccn2
Ccn3
mRNA Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
PBS + Scrambled Peptide
PBS + BLR-200
Lox
Plod2

Figure 3-8, Supplementary Figure 1. BLR-200 does not have any significant effect on normal healthy tissue.

Wild-type C57BL/6J mice were treated with PBS + scrambled peptide (n=6) or PBS + BLR-200 (n=6). **(A)** At the conclusion of the experiment, dermal tissue was fixed and stained with H&E. Representative images are shown. Dermal thickness measurements were taken at 3-6 random points in each section, averaged for each mouse, and compared (students t-test; *$p<0.05$). **(B)** Dermal tissue was stained with Masson's trichrome stain to examine histological changes in collagen organization. Images were taken at 3-6 random points in each section. Representative images are shown. The percent area of the dermal layer stained for trichrome was determined using ImageJ (student's t-test; *$p<0.05$). **(C)** Total RNA was extracted from the skin and subjected to SYBR Green real-time PCR using primers detecting *Ccn1*, *Ccn2*, *Ccn3*, *Lox*, and *Plod2*. Relative mRNA expression, compared to the housekeeping gene *Rn18s*, was determined using the ΔΔCt method (one-way ANOVA and Tukey's post-hoc test; *$p<0.05$).

3.6 References

Abd El Kader, T., Kubota, S., Janune, D., Nishida, T., Hattori, T., Aoyama, E., Perbal, B., Kuboki, T., & Takigawa, M. (2012). Anti-fibrotic effect of CCN3 accompanied by altered gene expression profile of the CCN family. *Journal of Cell Communication and Signaling*, *7*(1), 11–18. https://doi.org/10.1007/S12079-012-0180-4

Allanore, Y., Simms, R., Distler, O., Trojanowska, M., Pope, J., Denton, C. P., & Varga, J. (2015). Systemic sclerosis. *Nature Reviews Disease Primers*, *1*(15002), 1–21. https://doi.org/10.1038/nrdp.2015.2

Andenæs, K., Lunde, I. G., Mohammadzadeh, N., Dahl, C. P., Aronsen, J. M., Strand, M. E., Palmero, S., Sjaastad, I., Christensen, G., Engebretsen, K. V. T., & Tønnessen, T. (2018). The extracellular matrix proteoglycan fibromodulin is upregulated in clinical and experimental heart failure and affects cardiac remodeling. *PLoS ONE*, *13*(7), e0201422. https://doi.org/10.1371/JOURNAL.PONE.0201422

Armstrong, J. J. (2019). Modulation of Inflammation Driven Wound Healing after Glaucoma Surgery. *Electronic Thesis and Dissertation Repository*, 6534.

Belloli, L., Carlo-Stella, N., Ciocia, G., Chiti, A., Massarotti, M., & Marasini, B. (2008). Myocardial involvement in systemic sclerosis. *Rheumatology*. https://doi.org/10.1093/rheumatology/ken186

Brenner, M. C., Krzyzanski, W., Chou, J. Z., Signore, P. E., Fung, C. K., Guzman, D., Li, D., Zhang, W., Olsen, D. R., Nguyen, V.-T. L., Koo, C. W., Sternlicht, M. D., & Lipson, K. E. (2016). FG-3019, a Human Monoclonal Antibody Recognizing Connective Tissue Growth Factor, is Subject to Target-Mediated Drug Disposition. *Pharmaceutical Research*, *33*(8), 1849. https://doi.org/10.1007/S11095-016-1918-0

Chadli, L., Sotthewes, B., Li, K., Andersen, S. N., Cahir-McFarland, E., Cheung, M., Cullen, P., Dorjée, A., de Vries-Bouwstra, J. K., Huizinga, T. W. J., Fischer, D. F., DeGroot, J., Viney, J. L., Zheng, T. S., Aarbiou, J., & Gardet, A. (2019). Identification of regulators of the myofibroblast phenotype of primary dermal fibroblasts from early diffuse systemic sclerosis patients. *Scientific Reports*, *9*(1), 1–12. https://doi.org/10.1038/s41598-019-41153-w

Chen, C. C., Chen, N., & Lau, L. F. (2001). The Angiogenic Factors Cyr61 and Connective Tissue Growth Factor Induce Adhesive Signaling in Primary Human Skin Fibroblasts. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M008087200

Chen, C. C., & Lau, L. F. (2009). Functions and mechanisms of action of CCN matricellular proteins. *International Journal of Biochemistry and Cell Biology*. https://doi.org/10.1016/j.biocel.2008.07.025

Chen, L., Li, S., & Li, W. (2018). LOX/LOXL in pulmonary fibrosis: potential therapeutic targets. *Journal of Drug Targeting*, *27*(7), 790–796. https://doi.org/10.1080/1061186X.2018.1550649

Czirják, L., Foeldvari, I., & Müller-Ladner, U. (2008). Skin involvement in systemic sclerosis. *Rheumatology*. https://doi.org/10.1093/rheumatology/ken309

Darby, I., Skalli, O., & Gabbiani, G. (1990). Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *63*(1), 21–29. https://europepmc.org/article/med/2197503

di Carlo, S. E., & Peduto, L. (2018). The perivascular origin of pathological fibroblasts. *Journal of Clinical Investigation*, *128*(1), 54–63. https://doi.org/10.1172/JCI93558

Domogatskaya, A., Rodin, S., & Tryggvason, K. (2012). Functional diversity of laminins. *Annual Review of Cell and Developmental Biology*, *28*, 523–553. https://doi.org/10.1146/ANNUREV-CELLBIO-101011-155750

Dorn, L. E., Petrosino, J. M., Wright, P., & Accornero, F. (2018). CTGF/CCN2 is an autocrine regulator of cardiac fibrosis. *Journal of Molecular and Cellular Cardiology*, *121*, 205–211. https://doi.org/10.1016/J.YJMCC.2018.07.130

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., le Digabel, J., Forcato, M., Bicciato, S., Elvassore, N., & Piccolo, S. (2011). Role of YAP/TAZ in mechanotransduction. *Nature*, *474*(7350), 179–183. https://doi.org/10.1038/nature10137

Dziadzio, M., Usinger, W., Leask, A., Abraham, D., Black, C. M., Denton, C., & Stratton, R. (2005). N-terminal connective tissue growth factor is a marker of the fibrotic phenotype in scleroderma. *Monthly Journal of the Association of Physicians*. https://doi.org/10.1093/qjmed/hci078

Gabbiani, G. (2003). The myofibroblast in wound healing and fibrocontractive diseases. In *Journal of Pathology*. https://doi.org/10.1002/path.1427

Gardner, H., Shearstone, J. R., Bandaru, R., Crowell, T., Lynes, M., Trojanowska, M., Pannu, J., Smith, E., Jablonska, S., Blaszczyk, M., Tan, F. K., & Mayes, M. D. (2006). Gene profiling of scleroderma skin reveals robust signatures of disease that are imperfectly reflected in the transcript profiles of explanted fibroblasts. *Arthritis & Rheumatology*, *54*(6), 1961–1973. https://doi.org/10.1002/ART.21894

Gjaltema, R. A. F., de Rond, S., Rots, M. G., & Bank, R. A. (2015). Procollagen Lysyl Hydroxylase 2 Expression Is Regulated by an Alternative Downstream Transforming Growth Factor-1 Activation Mechanism *. *Journal of Biological Chemistry*, *290*(47), 28465–28476. https://doi.org/10.1074/jbc.M114.634311

Hinz, B. (2010). The myofibroblast: Paradigm for a mechanically active cell. *Journal of Biomechanics*. https://doi.org/10.1016/j.jbiomech.2009.09.020

Hinz, B. (2015). Myofibroblasts. In *Experimental Eye Research*. https://doi.org/10.1016/j.exer.2015.07.009

Hoberg, M., Rudert, M., Pap, T., Klein, G., Gay, S., & Aicher, W. K. (2007). Attachment to laminin-111 facilitates transforming growth factor β-induced expression of matrix metalloproteinase-3 in synovial fibroblasts. *Annals of the Rheumatic Diseases*, *66*(4), 451. https://doi.org/10.1136/ARD.2006.060228

Holmes, A. M., Ponticos, M., Shi-wen, X., Denton, C. P., & Abraham, D. J. (2011). Elevated CCN2 expression in scleroderma: a putative role for the TGFβ accessory receptors TGFβRIII and endoglin. *Journal of Cell Communication and Signaling*, *5*(3), 177. https://doi.org/10.1007/S12079-011-0140-4

Hung, C., Linn, G., Chow, Y.-H., Kobayashi, A., Mittelsteadt, K., Altemeier, W. A., Gharib, S. A., Schnapp, L. M., & Duffield, J. S. (2013). Role of Lung Pericytes and Resident Fibroblasts in the Pathogenesis of Pulmonary Fibrosis. *American Journal of Respiratory and Critical Care Medicine*, *188*(7), 820–830. https://doi.org/10.1164/RCCM.201212-2297OC

Jun, J.-I., & Lau, L. F. (2011). Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. *Nature Reviews Drug Discovery*, *10*(12), 945–963. https://doi.org/10.1038/nrd3599

Kalamajski, S., Bihan, D., Bonna, A., Rubin, K., & Farndale, R. W. (2016). Fibromodulin Interacts with Collagen Cross-linking Sites and Activates Lysyl Oxidase. *Journal of Biological Chemistry*, *291*(15), 7960. https://doi.org/10.1074/JBC.M115.693408

Kanaizuka, I., Sasaki, T., & Nakajima, H. (1991). Type IV collagen and laminin levels in the sera from patients with systemic scleroderma (PSS). *Japanese Journal of Dermatology*, *101*(8), 819–823.

King, J., Abraham, D., & Stratton, R. (2018). Chemokines in systemic sclerosis. *Immunology Letters*, *195*, 68–75. https://doi.org/10.1016/J.IMLET.2017.12.001

Kubota, S., & Takigawa, M. (2007). CCN family proteins and angiogenesis: from embryo to adulthood. *Angiogenesis*, *10*(1), 1–11. https://doi.org/10.1007/S10456-006-9058-5

Leask, A. (2013). CCN2: a novel, specific and valid target for anti-fibrotic drug intervention. *Expert Opinion on Therapeutic Targets*, *17*(9), 1067–1071. https://doi.org/10.1517/14728222.2013.812074

Leask, A. (2015). Matrix remodeling in systemic sclerosis. In *Seminars in Immunopathology*. https://doi.org/10.1007/s00281-015-0508-2

Leask, A., & Abraham, D. (2004). TGF-β signaling and the fibrotic response. *FASEB Journal*, *18*(7), 816–827. https://doi.org/10.1096/fj.03-1273rev

Leask, A., & Abraham, D. J. (2006). All in the CCN family: essential matricellular signaling modulators emerge from the bunker. *Journal of Cell Science*. https://doi.org/10.1242/jcs.03270

Leask, A., Holmes, A., Black, C. M., & Abraham, D. J. (2003). Connective tissue growth factor gene regulation: Requirements for its induction by transforming growth factor-β2 in fibroblasts. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M210366200

Lemaire, R., Farina, G., Bayle, J., Dimarzio, M., Pendergrass, S. A., Milano, A., Whitfield, M. L., & Lafyatis, R. (2010). Antagonistic Effect of the Matricellular Signaling Protein CCN3 on TGF-β- and Wnt-Mediated Fibrillinogenesis in Systemic

Sclerosis and Marfan Syndrome. *Journal of Investigative Dermatology*, *130*(6), 1514–1523. https://doi.org/10.1038/JID.2010.15

Liu, P., Chen, H., Yan, L., & Sun, Y. (2020). Laminin α5 modulates fibroblast proliferation in epidural fibrosis through the PI3K/AKT/mTOR signaling pathway. *Molecular Medicine Reports*, *21*(3), 1500. https://doi.org/10.3892/MMR.2020.10967

Liu, S., Herault, Y., Pavlovic, G., & Leask, A. (2014). Skin progenitor cells contribute to bleomycin-induced skin fibrosis. *Arthritis & Rheumatology*. https://doi.org/10.1002/art.38276

Liu, S., Shi-Wen, X., Abraham, D. J., & Leask, A. (2011). CCN2 is required for bleomycin-induced skin fibrosis in mice. *Arthritis & Rheumatology*, *63*(1), 239–246. https://doi.org/10.1002/art.30074

Lóránd, V., Czirják, L., & Minier, T. (2014). Musculoskeletal involvement in systemic sclerosis. *La Presse Médicale*, *43*(10), 315–328. https://doi.org/10.1016/j.lpm.2014.03.027

Makino, K., Makino, T., Stawski, L., Lipson, K. E., Leask, A., & Trojanowska, M. (2017). Anti-connective tissue growth factor (CTGF/CCN2) monoclonal antibody attenuates skin fibrosis in mice models of systemic sclerosis. *Arthritis Research & Therapy*, *19*(1), 134. https://doi.org/10.1186/s13075-017-1356-3

Meyringer, R., Neumann, E., Judex, M., Landthaler, M., Kullmann, F., Scholmerich, J., Gay, S., Tarner, I. H., Distler, O., & Müller-Ladner, U. (2007). Analysis of gene expression patterns in systemic sclerosis fibroblasts using RNA arbitrarily primed-polymerase chain reaction for differential display. *Journal of Rheumatology*, *34*(4), 747–753.

Mormone, E., Lu, Y., Ge, X., Fiel, M. I., & Nieto, N. (2012). Fibromodulin, an Oxidative Stress-Sensitive Proteoglycan, Regulates the Fibrogenic Response to Liver Injury in Mice. *Gastroenterology*, *142*(3), 621. https://doi.org/10.1053/J.GASTRO.2011.11.029

Nguyen, X.-X., Nishimoto, T., Takihara, T., Mlakar, L., Bradshaw, A. D., & Feghali-Bostwick, C. (2021). Lysyl oxidase directly contributes to extracellular matrix production and fibrosis in systemic sclerosis. *Lung Cellular and Molecular Physiology*, *320*(1), L29–L40. https://doi.org/10.1152/AJPLUNG.00173.2020

Parapuram, S. K., Thompson, K., Tsang, M., Hutchenreuther, J., Bekking, C., Liu, S., & Leask, A. (2015). Loss of PTEN expression by mouse fibroblasts results in lung fibrosis through a CCN2-dependent mechanism. *Matrix Biology*. https://doi.org/10.1016/j.matbio.2015.01.017

Perbal, B. (2004). CCN proteins: Multifunctional signalling regulators. In *Lancet*. https://doi.org/10.1016/S0140-6736(03)15172-0

Perbal, B. (2013). CCN proteins: A centralized communication network. *Journal of Cell Communication and Signaling*. https://doi.org/10.1007/s12079-013-0193-7

Perbal, B. (2018). The concept of the CCN protein family revisited: a centralized coordination network. *Journal of Cell Communication and Signaling*, *12*(1), 3. https://doi.org/10.1007/S12079-018-0455-5

Petz, M., Them, N., Huber, H., Beug, H., & Mikulits, W. (2012). La enhances IRES-mediated translation of laminin B1 during malignant epithelial to mesenchymal transition. *Nucleic Acids Research*, *40*(1), 302. https://doi.org/10.1093/NAR/GKR717

Phanish, M. K., Winn, S. K., & Dockrell, M. E. C. (2010). Connective Tissue Growth Factor-(CTGF, CCN2) – A Marker, Mediator and Therapeutic Target for Renal Fibrosis. *Nephron Experimental Nephrology*, *114*(3), e83–e92. https://doi.org/10.1159/000262316

Quesnel, K., Shi-wen, X., Hutchenreuther, J., Xiao, Y., Liu, S., Peidl, A., Naskar, D., Siqueira, W. L., O'Gorman, D. B., Hinz, B., Stratton, R. J., & Leask, A. (2019). CCN1 expression by fibroblasts is required for bleomycin-induced skin fibrosis. *Matrix Biology Plus*, *3*, 100009. https://doi.org/10.1016/J.MBPLUS.2019.100009

Rachfal, A. W., & Brigstock, D. R. (2003). Connective tissue growth factor (CTGF/CCN2) in hepatic fibrosis. *Hepatology Research*, *26*(1), 1–9. https://doi.org/10.1016/S1386-6346(03)00115-3

Rajkumar, V. S., Howell, K., Csiszar, K., Denton, C. P., Black, C. M., & Abraham, D. J. (2005). Shared expression of phenotypic markers in systemic sclerosis indicates a convergence of pericytes and fibroblasts to a myofibroblast lineage in fibrosis. *Arthritis Research & Therapy*, *7*(5), R1123. https://doi.org/10.1186/AR1790

Rigau, A. R., Luber, M., & Distler, J. H. (2021). Mouse Models of Skin Fibrosis. *Methods in Molecular Biology*, *2299*, 371–383. https://doi.org/10.1007/978-1-0716-1382-5_25

Riser, B. L., Najmabadi, F., Garchow, K., Barnes, J. L., Peterson, D. R., & Sukowski, E. J. (2014). Treatment with the Matricellular Protein CCN3 Blocks and/or Reverses Fibrosis Development in Obesity with Diabetic Nephropathy. *American Journal of Pathology*, *184*(11), 2908–2921. https://doi.org/10.1016/j.ajpath.2014.07.009

Riser, B. L., Najmabadi, F., Perbal, B., Peterson, D. R., Rambow, J. A., Riser, M. L., Sukowski, E., Yeger, H., & Riser, S. C. (2009). CCN3 (NOV) is a negative regulator of CCN2 (CTGF) and a novel endogenous inhibitor of the fibrotic pathway in an in vitro model of renal disease. *American Journal of Pathology*, *174*(5), 1725–1734. https://doi.org/10.2353/ajpath.2009.080241

Riser, B. L., Najmabadi, F., Perbal, B., Rambow, J. A., Riser, M. L., Sukowski, E., Yeger, H., Riser, S. C., & Peterson, D. R. (2010). CCN3/CCN2 regulation and the fibrosis of diabetic renal disease. *Journal of Cell Communication and Signaling*, *4*(1), 39. https://doi.org/10.1007/s12079-010-0085-z

Robinson, M.D., McCarthy, D.J., Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics, 26(1), 139-140. https://doi.org/10.1093/bioinformatics/btp616

Santos, V. N. dos, Leite-Mór, M. M. B., Kondo, M., Martins, J. R., Nader, H., Lanzoni, V. P., & Parise, E. R. (2005). Serum laminin, type IV collagen and hyaluronan as fibrosis markers in non-alcoholic fatty liver disease. *Brazilian Journal of Medical and Biological Research*, *38*(5), 747–753. https://doi.org/10.1590/S0100-879X2005000500012

Schulz, J. N., Plomann, M., Sengle, G., Gullberg, D., Krieg, T., & Eckes, B. (2018). New developments on skin fibrosis - Essential signals emanating from the extracellular matrix for the control of myofibroblasts. *Matrix Biology*, *68–69*, 522–532. https://doi.org/10.1016/J.MATBIO.2018.01.025

Shi-wen, X., Racanelli, M., Ali, A., Simon, A., Quesnel, K., Stratton, R. J., & Leask, A. (2021). Verteporfin inhibits the persistent fibrotic phenotype of lesional scleroderma dermal fibroblasts. *Journal of Cell Communication and Signaling*, *15*(1), 71. https://doi.org/10.1007/S12079-020-00596-X

Sonnylal, S., Shi-Wen, X., Leoni, P., Naff, K., van Pelt, C. S., Nakamura, H., Leask, A., Abraham, D., Bou-Gharios, G., & de Crombrugghe, B. (2010). Selective Expression of Connective Tissue Growth Factor in Fibroblasts In Vivo Promotes Systemic Tissue Fibrosis. *Arthritis & Rheumatology*, *62*(5), 1–17. https://doi.org/10.1002/art.27382

Sun, Y., Wang, T. L., Toh, W. S., & Pei, M. (2017). The role of laminins in cartilaginous tissues: from development to regeneration. *European Cells & Materials*, *34*, 54. https://doi.org/10.22203/ECM.V034A03

Talior-Volodarsky, I., Connelly, K. A., Arora, P. D., Gullberg, D., & McCulloch, C. A. (2012). α11 integrin stimulates myofibroblast differentiation in diabetic cardiomyopathy. *Cardiovascular Research*, *96*(2), 265–275. https://doi.org/10.1093/CVR/CVS259

Thompson, K., Murphy-Marshman, H., & Leask, A. (2014). ALK5 inhibition blocks TGFβ-induced CCN1 expression in human foreskin fibroblasts. *Journal of Cell Communication and Signaling*, *8*(1), 59. https://doi.org/10.1007/s12079-014-0229-7

Toyama, T., Looney, A. P., Baker, B. M., Stawski, L., Haines, P., Simms, R., Szymaniak, A. D., Varelas, X., & Trojanowska, M. (2018). Therapeutic targeting of TAZ and YAP by dimethyl fumarate in systemic sclerosis fibrosis. *Journal of Investigative Dermatology*, *138*(1), 88. https://doi.org/10.1016/J.JID.2017.08.024

Tsang, M., & Leask, A. (2014). CCN2 is required for recruitment of Sox2-expressing cells during cutaneous tissue repair. *Journal of Cell Communication and Signaling*, *9*(4), 341–346. https://doi.org/10.1007/S12079-014-0245-7

Tsang, M., Quesnel, K., Vincent, K., Hutchenreuther, J., Postovit, L.-M., & Leask, A. (2019). Insights into Fibroblast Plasticity: Cellular Communication Network 2 Is Required for Activation of Cancer-Associated Fibroblasts in a Murine Model of Melanoma. *American Journal of Pathology*, *190*(1), 206–221. https://doi.org/10.1016/J.AJPATH.2019.09.006

van der Slot, A. J., Zuurmond, A.-M., Bardoel, A. F. J., Wijmenga, C., Pruijs, H. E. H., Sillence, D. O., Brinckmann, J., Abraham, D. J., Black, C. M., Verzijl, N., DeGroot, J., Hanemaaijer, R., TeKoppele, J. M., Huizinga, T. W. J., & Bank, R. A. (2003). Identification of PLOD2 as Telopeptide Lysyl Hydroxylase, an Important Enzyme in Fibrosis *. *Journal of Biological Chemistry*, *278*(42), 40967–40972. https://doi.org/10.1074/JBC.M307380200

Varga, J., & Abraham, D. (2007). Systemic sclerosis: a prototypic multisystem fibrotic disorder. *Journal of Clinical Investigation*, *117*(3), 557–567. https://doi.org/10.1172/JCI31139

Woodfin, A., Voisin, M.-B., & Nourshargh, S. (2007). PECAM-1: A Multi-Functional Molecule in Inflammation and Vascular Biology. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *27*(12), 2514–2523. https://doi.org/10.1161/ATVBAHA.107.151456

Yamada, M., Hirabayashi, K., Kawanishi, A., Hadano, A., Takanashi, Y., Izumi, H., Kawaguchi, Y., Mine, T., Nakamura, N., & Nakagohri, T. (2017). Nectin-1 expression in cancer-associated fibroblasts is a predictor of poor prognosis for pancreatic ductal adenocarcinoma. *Surgery Today*, *48*(5), 510–516. https://doi.org/10.1007/S00595-017-1618-3

Yamamoto, T. (2006). The bleomycin-induced scleroderma model: what have we learned for scleroderma pathogenesis? *Archives of Dermatological Research*, *297*(8), 333–344. https://doi.org/10.1007/S00403-005-0635-Z

Yamamoto, T., & Katayama, I. (2011). Vascular Changes in Bleomycin-Induced Scleroderma. *International Journal of Rheumatology*, *2011*, 1–5. https://doi.org/10.1155/2011/270938

Yamamoto, T., Takagawa, S., Katayama, I., Yamazaki, K., Hamazaki, Y., Shinkai, H., & Nishioka, K. (1999). Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. *Journal of Investigative Dermatology*. https://doi.org/10.1046/j.1523-1747.1999.00528.x

Zeltz, C., Alam, J., Liu, H., Erusappan, P. M., Hoschuetzky, H., Molven, A., Parajuli, H., Cukierman, E., Costea, D. E., Lu, N., & Gullberg, D. (2019). α11β1 integrin is induced in a subset of cancer- associated fibroblasts in desmoplastic tumor stroma and mediates in vitro cell migration. *Cancers*, *11*(6), 765. https://doi.org/10.3390/CANCERS11060765

Chapter 4

4 Single-cell RNA-sequencing reveals that therapeutic peptides based on CCN3 impair early inflammatory and fibrotic changes in a mouse model of systemic sclerosis

4.1 Introduction

Fibrotic conditions are characterized by the excessive deposition of extracellular matrix (ECM) components, often due to dysregulation of the normal wound healing process, leading to pathological scarring that can result in organ failure and, ultimately, death (Schulz et al., 2018; Walraven & Hinz, 2018). An example of such a fibrotic condition is the autoimmune connective tissue disorder systemic sclerosis (SSc). Patients with SSc often experience progressive fibrosis of the skin and internal organs, including the lung, heart, gastrointestinal tract, and kidneys (Leask, 2015). The mortality rates associated with SSc are frequently high, and patients will often die from lung fibrosis (Denton et al., 2018). There is currently no approved therapy for SSc, in part due to its unknown etiology, highlighting the need to further characterize the mechanism underlying disease. Since SSc shares similar features with other fibrotic disorders, understanding the molecular basis of SSc may reveal pertinent information regarding pathological fibrosis in general.

In SSc patients, a notable early pathological change in clinically involved connective tissue is vascular injury, which leads to infiltration of immune mediators, such as activated T-cells and macrophages (Allanore et al., 2015; Cutolo et al., 2019). Infiltration of these immune cells results in the release of several pro-inflammatory and pro-fibrotic cytokines, including interleukin-1 (IL-1), IL-6, and transforming growth factor β (TGFβ) isoforms (King et al., 2018; Muangchan & Pope, 2012). These cytokines promote a pro-fibrotic microenvironment which activates keratinocytes and fibroblasts, leading to fibrosis (King et al., 2018; Russo et al., 2021).

The critical responder cell type responsible for fibrogenesis, including in SSc, is a particular type of activated fibroblast, termed the myofibroblast (Chen et al., 2005; Gabbiani, 2003; Hinz, 2010, 2015). One of the defining features of activated myofibroblasts is the expression of the highly contractile protein α-smooth muscle actin

(αSMA) (Gabbiani, 2003). Once activated, myofibroblasts will excessively synthesize ECM components such as type I collagen and fibronectin, as well as adhere to and contract the surrounding ECM (Hinz, 2015). Although myofibroblast differentiation is required for normal wound repair, in this process myofibroblasts disappear, due to apoptosis, after wound closure (Desmoulière et al., 1995). Conversely, in pathological fibrosis, myofibroblasts persist. As immune infiltration causes fibroblasts to differentiate into myofibroblasts, studying how fibroblasts respond to the inflammatory microenvironment should provide valuable contributions to our overall understanding of fibrogenesis.

The Cellular Communication Network (CCN) family of matricellular proteins is the subject of many recent studies, as their collective action has been shown to contribute to fibrogenesis (Leask, 2020b). In general, CCN proteins play a role in modulating cellular responses to the local microenvironment, by directly binding to adhesion receptors, such as integrins, as well as by acting as co-factors for other regulatory molecules (Lau, 2016; Leask, 2020b). The wide array of actions mediated by CCN proteins depends on the presence of the proteins and receptors with which they interact (Leask, 2020b). Therefore, their contribution to pathological conditions, such as fibrosis, is extremely context- and stage-specific, specifically integrating signals that are occurring in a specific location, at a specific point in time.

CCN2 is induced in differentiated myofibroblasts during wound healing and is widely accepted as a marker of myofibroblasts in pathological fibrosis (Perbal, 2018). Furthermore, CCN2 expression by fibroblasts is required for fibrosis in multiple mouse models of SSc (Liu et al., 2011, 2013). CCN1 expression by fibroblasts is also required for dermal fibrosis in mouse models, and likely plays an important role in collagen organization (Quesnel et al., 2019; Shi-wen et al., 2021). Of clinical importance, Both CCN1 and CCN2 expression are increased in the fibrotic lesions of early onset diffuse SSc patients, providing evidence that these proteins may play crucial roles in SSc fibrosis (Holmes et al., 2011). Furthermore, CCN1 and CCN2 deletion from fibroblasts had no significant effects on the normal wound healing process (Liu et al., 2014; Quesnel et al., 2019). Considering these specific effects of CCN1 and CCN2 on fibrosis, targeting these proteins may represent a potential anti-fibrotic approach.

Another member of the CCN family, CCN3, is reciprocally regulated to CCN1 and CCN2 in several cell types, often exhibiting opposing functional behaviour; thus, CCN3 may act as an endogenous antagonist of the pro-fibrotic activity of CCN1 and CCN2 (Abd El Kader et al., 2012; Lemaire et al., 2010; Perbal, 2018; Riser et al., 2015). For example, treatment with recombinant human CCN3 blocks and reverses fibrosis in a mouse model of diabetic nephropathy (Riser et al., 2009, 2014). These observations culminated in the development of a proprietary CCN3-derived peptide (BLR-200) which mimicked the *in vivo* anti-fibrotic ability of intact, full-length CCN3. According to an issued patent, BLR-200 blocks mesangial cell adhesion to CCN2 and inhibits the ability of pro-fibrotic agents to provoke collagen expression in dermal fibroblasts *in vitro* (U.S. Patent No. 9114112B2, 2015). Therefore, BLR-200 may represent a novel therapeutic option for SSc.

In my previous chapter, I showed that BLR-200 treatment prevents progressive dermal fibrosis in the bleomycin-induced model of SSc. This model mimics the initial stages of SSc dermal fibrosis, histologically and biochemically, and thus is useful to study fibrogenesis (Yamamoto, 2017; Yamamoto et al., 1999). Repeated subcutaneous injection of bleomycin causes a short-lived inflammatory response that leads to progressive fibrosis after 3-4 weeks (Yamamoto, 2006). I have shown that BLR-200 prevents fibrotic changes after 28 days. However, the effect of BLR-200 on the response of fibroblasts to inflammation, prior to the development of fibrosis, remains to be determined.

Previous studies employing standard analytical techniques aimed at understanding the central mechanisms that drive pathological fibrosis have shown limited progress. These limitations are largely due to the complexity and heterogeneity of tissue changes that occur during fibrogenesis. However, the use of unbiased single-cell RNA-sequencing (scRNA-seq) may offer the potential to overcome such limitations. Recent studies have shown that scRNA-seq analysis of fibroblast heterogeneity in fibrosis provides useful information on the diversity of aberrant cell populations present in fibrotic lesions (Deng et al., 2021; Vorstandlechner et al., 2020). Furthermore, unbiased transcriptomic analysis could further our understanding of key mechanistic mediators of fibrosis.

In the experiments reported in this chapter, I employ unbiased scRNA-seq and proteomic analysis to investigate how fibroblasts respond to the inflammatory microenvironment in bleomycin-induced dermal fibrosis at day 10, and determine if the CCN3-derived peptide, BLR-200, can modify this response. To study this fibroblast response, I will use transgenic mice to isolate and sequence COL1A2-expressing dermal fibroblasts (Bou-Gharios et al., 1996; Denton et al., 2001). This collagen-expressing fibroblast population has been shown to contribute to bleomycin-induced dermal fibrosis in a CCN2-dependent manner (Liu et al., 2014, Tsang et al., 2019). My aim is to characterize the contribution of these collagen-expressing resident dermal fibroblasts to the heterogenous fibroblast subpopulation in response to the pro-inflammatory stage of bleomycin-induced fibrosis. Understanding the heterogenous fibroblast population in fibrotic disease, and how it responds to an anti-fibrotic treatment such as BLR-200, will provide invaluable insight into the overall process of fibrogenesis.

4.2 Methods

4.2.1 Bleomycin-Induced Model of Dermal Fibrosis

Bleomycin sulfate (0.1 units/100 ml per injection; Sigma) or vehicle (PBS, 100 ml per injection) was injected subcutaneously into a single location on the flank of genetically modified *Col1a2-Cre(ER)T;Rosa26mTmG* C57BL/6J mice once daily for 10 days. Bleomycin-treated mice were further divided into two treatment groups, which were injected intraperitoneally 3 times per week with either scrambled peptide (10 µg/kg) or BLR-200 (10 µg/kg). At the end of the treatment period, the mice were sacrificed via CO_2 inhalation. Skin samples were collected for proteomic analysis, and fibroblasts were isolated from the skin of mice for single-cell RNA-sequencing analysis. All animal protocols were approved by the Animal Care and Veterinary Services at Western University.

4.2.2 Generation of Genetically Modified *Col1a2-Cre(ER)T;Rosa26mTmG* Mice for Fibroblast Isolation and Proteomic Analysis

Mice were hemizygous for tamoxifen-dependent Cre recombinase under the control of the fibroblast specific proα2 collagen promoter (*Col1a2-Cre(ER)-T*) (Denton et al., 2001) and homozygous for a double fluorescent reporter transgene (*mT/mG*) integrated into the *Gt(ROSA)26Sor* locus (Jackson Laboratories) that results in the expression of membrane-targeted tdTomato (RFP) prior to Cre-mediated excision and membrane-targeted GFP after excision. This genetically modified model has been extensively described in previous publications (Liu & Leask, 2013; Tsang et al., 2019). The *Col1a2-Cre(ER)-T* mice possess a fibroblast-specific enhancer, initially identified by the laboratory of Benoit de Crombrugghe (Bou-Gharios et al., 1996), subcloned upstream of the *Col1a2* minimal promoter. This construct permits transgene expression specifically in fibroblasts and not in other type I collagen-expressing cells (Ponticos et al., 2004).

Polymerase chain reaction and agarose gel electrophoresis was used to genotype the DNA of the experimental animals for appropriate expression of *Cre* and *mTmG*, as previously described (Liu et al., 2013; Tsang et al., 2019). To induce membrane-targeted GFP expression in *Col1a2*-expressing cells, 3-week-old mice were injected intraperitoneally with a tamoxifen suspension (0.1 mL of 10 mg/mL 4-hydroxitamoxifen, Sigma) over 5 days. The mice were then subjected to bleomycin-induced fibrosis as described above in Section 4.2.1.

4.2.3 Isolation of Fibroblasts

To isolate fibroblasts from the skin of treated mice, skin samples from the injection site were incubated in a solution of 2 mg/mL collagenase (Gibco) in DMEM (Thermo Fisher) for 3 hours at 37°C. The connective tissue layer was then scraped off, added to 5mL DMEM and mixed until homogenous. The solution was then centrifuged for 1 minute at 500 rpm. The supernatant layer containing cells of the connective tissue layer was then collected and centrifuged for 5 minutes at 2000 rpm. The supernatant was removed, and the cell pellet was then resuspended in a solution of 3% FBS (Gibco) in PBS and filtered through a 40 µm cell strainer (Sigma). The filtered solution was centrifuged for 5 minutes

at 2000 rpm, the supernatant was discarded, and the cell pellet was resuspended in ammonium-chloride-potassium (ACK) buffer (Thermo Fisher) and incubated on ice for 10 minutes, to remove red blood cells. The cells were then centrifuged for 5 minutes at 2000 rpm and washed twice with 3% FBS in PBS, before being stained with SYTOX Blue (Thermo Fisher) as per the manufacturer's instructions. $30x10^6$ cells/mL were then subjected to fluorescence activated cell sorting (FACS) using a FACSAria III cell sorter (BD Biosciences) at the London Regional Flow Cytometry Facility. Forward- and side-scatter were used to select individual cells. A 405 nm laser was then used to select cells negative for SYTOX Blue. The remaining cells were sorted into *Col1a2*-lineage-positive (488 nm) and *Col1a2*-lineage-negative (561 nm) based on expression of GFP and tdTomato, respectively. *Col1a2*-lineage-positive cells (GFP+) were frozen down at -80°C in 100% DMSO (Sigma), then sent away for single-cell RNA-sequencing analysis.

4.2.4 Single-cell RNA-Sequencing Analysis

Col1a2-lineage-positive (GFP+) fibroblasts from our experiment were sent for single-cell RNA-sequencing by the Princess Margaret Genomics Center at the University of Toronto. Samples were prepared as outlined in 10X protocol (10X Genomics). Cells were thawed, washed, counted, and suspended in PBS (Life Technologies) +0.04% BSA (Miltenyi). Sample viability and cell counting was performed using a haemocytometer (Thermo Fisher). Following counting, the appropriate volume for each sample was calculated for a target capture of 2000 cells and loaded onto a 10x single cell B chip. After droplet generation, samples were transferred onto a pre-chilled 96 well plate (Eppendorf), heat sealed and incubated overnight in a Veriti 96-well thermocycler (Thermo Fisher). The next day, sample cDNA was recovered using Recovery Agent provided by 10x and subsequently cleaned up using a Silane DynaBead (Thermo Fisher) mix as outlined by the manufacturer's instructions. Purified cDNA was amplified for 12 cycles before being cleaned up using SPRIselect beads (Beckman Coulter). Samples were diluted 9:1 (elution buffer (Qiagen):cDNA) and run on a Bioanalyzer (Agilent Technologies) to determine cDNA concentration. cDNA libraries were prepared as outlined by the Single Cell 3' Reagent Kits v3 user guide with modifications to the PCR cycles based on the calculated cDNA concentration.

The molarity of each library was calculated based on library size as measured by the bioanalyzer and qPCR amplification data (Sigma). Samples were pooled and normalized to 10 nM, then diluted to 2 nM using elution buffer with 0.1% Tween20 (Sigma). Each 2 nM pool was denatured using 0.1 M NaOH at equal volumes for 5 minutes at room temperature. Library pools were further diluted to 20 pM using HT-1 (Illumina) before being diluted to a final loading concentration of 14 pM. 150 ul from the 14 pM pool was loaded into each well of an 8-well strip tube and loaded onto a cBot (Illumina) for cluster generation. Samples were sequenced on a HiSeq 2500 with the following run parameters: Read 1 – 26 cycles, read 2 – 98 cycles, index 1 – 8 cycles.

Approximately 2000 cells were captured from each sample and subjected to 30-50,000 reads per cell. Data filtering and analysis was performed using Cell Ranger (10X Genomic, v2.1.0) and Loupe Browser Software (10X Genomics, v5.1). Genes expressed in fewer than 5 cells were removed from analysis and cells were removed if they expressed fewer than 300 genes. Data were normalized to 10,000 UMI/cell and converted to log scale. Using Loupe Browser software, a set of the most variable genes were identified, and principal component analysis (PCA) was then performed to generate Uniform Manifold Approximation and Projection for dimension reduction (UMAP) plots to visualize cell populations based on similar gene expression. Gene Ontology (GO) analysis was performed on the top 100 most representative genes per cluster, which were queried into the Gene Functional Annotation Tool from DAVID Bioinformatics Database (v6.8). The top GO terms with a p-value < 0.05 were chosen to analyze fibroblast subpopulations, consistent with previously published data (Ascensión et al., 2021; Deng et al., 2021; Solé-Boldo et al., 2020; Vorstandlechner et al., 2020). Loupe Browser software was also used to generate violin plots showing the Log2 Max Count for genes in each cluster. Specific genes known to be involved in dermal fibrosis and the inflammatory response were examined.

4.2.5 Proteomic Analysis

Proteomic analysis was carried out in collaboration with colleagues from the Proteomic Resource Facility (PRF) in the Department of Pathology at the University of Michigan, where mass spectrometry-based Tandem Mass Tag (TMT, Thermo Fisher) was employed.

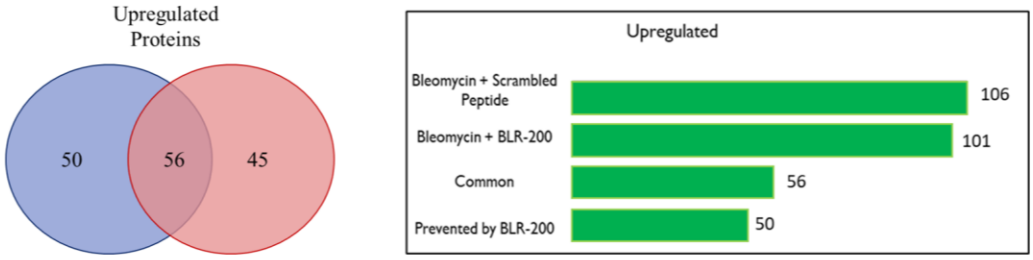
Full skin protein samples were homogenized and digested with trypsin. Protein fragments were individually labeled with one of ten isobaric mass tags following the manufacturer's protocol. After labelling, equal amounts of peptide from each condition were mixed. The labelled proteins were then fractionated by 2D-liquid chromatography, using basic pH reverse-phase separation followed by acidic pH reverse-phase. The samples were analyzed on a high-resolution, tribrid mass spectrometer (Orbitrap Fusion Tribrid, Thermo Fisher) using conditions optimized by the PRF. MultiNotch MS3 approach was employed to obtain accurate quantitation of the identified proteins. Data analysis was performed using Proteome Discoverer (v 2.3, Thermo Fisher). MS2 spectra were searched against the SwissProt reviewed mouse protein database (downloaded on 2019-06-29) using the following search parameters: MS1 and MS2 tolerance were set to 10 ppm and 0.6 Da, respectively; carbamidomethylation of cysteines (57.02146 Da) and TMT labeling of lysine and N-termini of peptides (229.16293 Da) were considered static modifications; oxidation of methionine (15.9949 Da) and deamidation of asparagine and glutamine (0.98401 Da) were considered variable. Identified proteins and peptides were filtered to retain only those that passed ≤2% false-discovery rate (FDR) threshold of detection. Quantitation was performed using reporter ion intensity extracted from high-quality MS3 spectra within a ±10 PPM window centered on the theoretical m/z value of each reporter ion. Reporter ion intensities were corrected for isotopic impurities of different TMT reagents as specified by the manufacturer. Only those peptide reporter ion intensities with an average signal-to-noise ratio of 9 and <40% co-isolation interference were considered for quantification. Differential protein expression between conditions, normalizing to control (PBS) for each subject's specimens separately was established using edgeR (Robinson et al., 2010). Then, results for individual proteins from six mice per treatment were pooled. Fold change ratios were produced for either bleomycin + scrambled mice or bleomycin + BLR-200 mice, relative to PBS. Differentially expressed proteins were filtered based on a ±1.8-fold cut-off. Reactome V69 (reactome.org) was used for pathway enrichment analyses.

4.3 Results

4.3.1 Proteomic analysis reveals that BLR-200 treatment prevents early fibrotic changes in bleomycin-induced fibrosis

To assess the ability of BLR-200 to suppress pro-fibrotic protein expression in the initial, inflammatory stages of bleomycin-induced fibrosis, I employed an unbiased proteomic approach. C57BL/6 mice were subjected to bleomycin-induced skin fibrosis, either in the presence of a scrambled control peptide (BLM), or BLR-200 (BLR-200). Ten days after the initiation of bleomycin injection, protein was extracted from full skin and subjected to tandem mass-tag spectrometry. Protein expression for BLM mice or BLR-200 mice was normalized to protein expression in control PBS mice, producing fold change differences. A list of upregulated proteins (>1.8-fold cut-off) in each experimental group was generated. Out of the upregulated proteins in BLM mice, 50/106 were prevented by BLR-200 treatment (Figure 4-1A). Proteins induced in BLM mice, but not BLR-200 mice were analyzed using the Reactome pathway database. It was found that several pathways involved in keratinocyte activation and myofibroblast contraction were prevented by BLR-200, including formation of the cornified envelope (R-MMU-6809371), keratinization (R-MMU-6805567), and smooth muscle contraction (R-MMU-445355) (Figure 4-1B). Further analysis of specific proteins revealed that BLR-200 prevented several keratins known to be involved in epithelial activation (Figure 4-1C). Keratinocytes are upregulated in the early stages of hyperproliferative disorders including in SSc (Aden et al., 2008; Nikitorowicz-Buniak et al., 2014) and their activation leads to production of pro-fibrotic cytokines, thereby influencing the activation of local fibroblasts (Coulombe, 1997; Lane & McLean, 2004). Keratin 1, 14, 16, and 6 are also upregulated in the epidermis of fibrotic lesions in SSc patients and keratinocyte-fibroblast interactions are abnormal in SSc (McCoy et al., 2017; Russo et al., 2021). Several protein markers of early fibrogenesis were also prevented by BLR-200 treatment (Figure 4-1D). Myosin light chain 6b and tropomyosin 3 contribute to transformation of fibroblasts into myofibroblasts (Fujimura et al., 2011; Malmstrom et al., 2004). Fibrillin-1 and dermatopontin are involved in cell-matrix interactions and play important roles in ECM formation and organization (Kissin et al., 2002; Okamoto & Fujiwara, 2009). Collectively, these data suggest that BLR-200 can suppress early, inflammatory-driven changes in bleomycin-induced dermal fibrosis.

A



B

Pathway identifier	Pathway name	Entities pValue
R-MMU-6809371	Formation of the cornified envelope	9.99E-16
R-MMU-6805567	Keratinization	4.57E-12
R-MMU-1266738	Developmental Biology	2.16E-07
R-MMU-446107	Type I hemidesmosome assembly	0.001619
R-MMU-2022377	Metabolism of Angiotensinogen to Angiotensins	0.003367
R-MMU-445355	Smooth Muscle Contraction	0.01129
R-MMU-1307965	betaKlotho-mediated ligand binding	0.015847
R-MMU-1433557	Signaling by SCF-KIT	0.020322
R-MMU-8957275	Post-translational protein phosphorylation	0.024
R-MMU-5661270	Formation of xylulose-5-phosphate	0.026274
R-MMU-381426	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	0.027312
R-MMU-72187	mRNA 3'-end processing	0.036054
R-MMU-5649702	APEX1-Independent Resolution of AP Sites via the Single Nucleotide Replacement Pathway	0.036593
R-MMU-189085	Digestion of dietary carbohydrate	0.036593

C Markers of epithelial activation

Protein	Abundance Ratio (BLM:PBS)	Abundance Ratio (BLR-200:PBS)
Keratin 16	12.634	0.968
Keratin 6	5.842	0.828
Keratin 5	3.399	1.155
Keratin 14	2.788	1.107
Keratin 1	2.341	0.903
Fillagrin	2.277	1.524
Hornerin	2.033	1.492

D Markers of early fibrogenesis/myofibroblast activation

Protein	Abundance Ratio (BLM:PBS)	Abundance Ratio (BLR-200:PBS)
Myosin light chain 6b	2.385	1.542
Fibrillin-1	2.380	1.149
Tropomyosin 3	1.869	1.291
Dermatopontin	1.813	1.564

Figure 4-1. BLR-200 prevents early fibrotic changes in the proteome of bleomycin-induced dermal fibrosis.

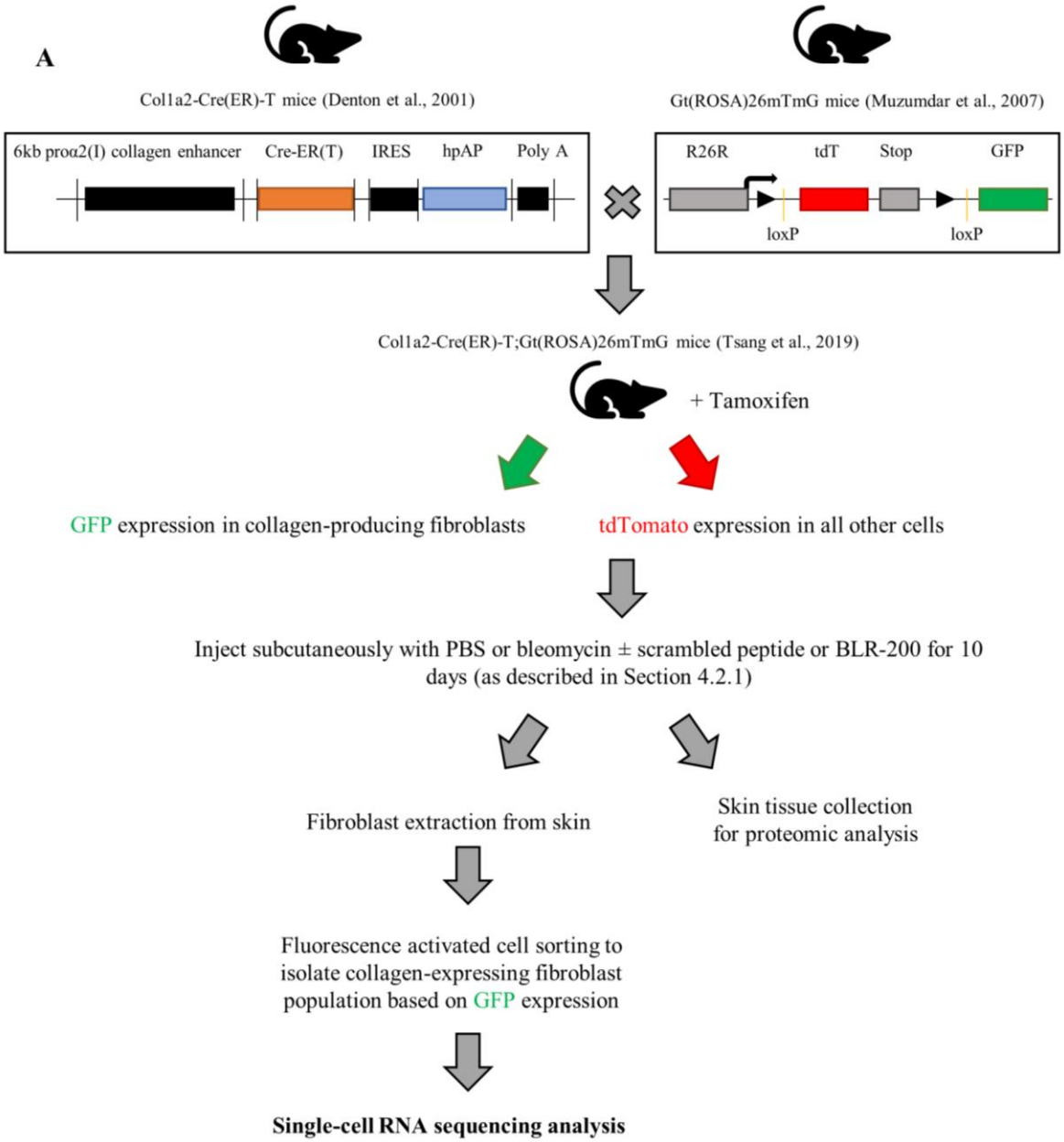
Mice were treated with either PBS (n=3), bleomycin + scrambled peptide (BLM; n=3) or bleomycin + BLR-200 (BLR-200; n=3). Total protein was extracted from the skin and subjected to tandem mass-tag mass spectrometry. Relative protein expression for BLM mice and BLR-200 mice was normalized to the control PBS group, generating fold change differences for all detected proteins. A list of upregulated proteins in each experimental group was generated using a 1.8-fold cut-off. **(A)** Venn diagram showing that 50 out of 106 proteins induced in BLM mice are prevented by BLR-200 treatment. **(B)** Reactome pathways analysis of bleomycin-induced proteins that were prevented by BLR-200 treatment shows that the top pathways prevented involve keratinocyte activation and smooth muscle contraction. **(C)** Analysis of fold change differences for proteins that were prevented by BLR-200 treatment. Markers of epithelial activation (keratin 16, keratin 6, keratin 5, keratin 14, keratin 1, fillagrin, and hornerin), markers of early fibrogenesis (fibrillin-1 and dermatopontin) and myofibroblast activation (myosin light chain 6b and tropomyosin 3) were prevented by BLR-200 treatment.

4.3.2 Single-cell RNA-sequencing reveals fibroblast heterogeneity in the fibrotic lesion of experimental mice

To further analyze if BLR-200 suppressed the early, inflammatory stage of bleomycin-induced fibrosis, I assessed if BLR-200 affected the ability of fibroblasts to be activated in response to the inflammatory microenvironment. Previously, our lab has shown that collagen-lineage fibroblasts were required for fibrogenesis (Liu, et al., 2014; Tsang et al., 2019; Tsang & Leask, 2014). To begin to address if BLR-200 could affect activation, in response to inflammation, of collagen-lineage fibroblasts, scRNA-seq was employed to first identify the collagen-linage fibroblasts subpopulations that are activated in response to inflammation. I labelled collagen-expressing synthetic fibroblasts with GFP using a fibroblast specific *Col1a2*-derived promoter/enhancer (Bou-Gharios et al., 1996). Three-week-old *Col1a2-Cre(ER)-T;Gt(ROSA)26mTmG* mice were injected with tamoxifen to activate Cre, enabling expression of the GFP reporter in collagen-lineage fibroblasts, and mice were subsequently subjected to bleomycin-induced skin fibrosis in the presence of BLR-200 (BLR-200) or scrambled peptide (BLM) (Figure 4-2). After 10 days of treatment, mice were sacrificed, and fibroblasts were extracted from the dorsal skin. Fluorescence activated cell sorting was then used to isolate GFP-expressing collagen-lineage fibroblasts. Isolated collagen-lineage fibroblasts were then subjected to scRNA-seq analysis. Quality control steps were performed by Cell Ranger to remove low quality cells (as described in Methods 4.2.5), and the transcriptomes of approximately 2000 cells from each treatment group were obtained.

In my initial analysis, unsupervised Uniform Manifold Approximation and Projection (UMAP)-clustering revealed that collagen-lineage fibroblasts gave rise to 7 cell clusters with distinct expression profiles (Figure 4-3A). It has previously been reported that, in human dermal tissue, fibroblasts can be divided into four main subpopulations based on known marker genes and functional annotations: secretory-papillary, secretory-reticular, mesenchymal, and inflammatory (Deng et al., 2021; Solé-Boldo et al., 2020). Based on these previous reports, I compared known markers with the most representative genes in each of cluster (Supplemental Files) and used Gene Ontology (GO) functional pathway analysis (Supplementary Figure 4-1A-C) to identify the distinct fibroblast subpopulations.

In the treatment groups, I identified two inflammatory fibroblast subpopulations (IF1, IF2), three secretory-reticular fibroblast subpopulations (SC1, SC2, SC3), and one mesenchymal fibroblast subpopulation (MES) (Figure 4-3A). One cell cluster in each treatment group had high expression of markers for mitochondrial interference and were classified as "unknown". The inflammatory fibroblast subpopulations expressed high levels of inflammatory markers C-X-C motif chemokine ligand 13 (*Cxcl13*), colony stimulating factor 2 (*Csf2*), and interleukin-33 (*Il33*) (Figure 4-3B). GO functional pathway analysis for these populations showed enriched cytokine- and chemokine-mediated signaling, neutrophil chemotaxis, positive regulation of chemokine production, and positive regulation of prostaglandin secretion (Supplementary Figure 4-1A-C). The reticular secretory fibroblast subpopulations expressed high levels of secretory markers matrix Gla protein (*Mgp*) and sparc-like 1 (*Sparcl1*) (Figure 3C). GO functional pathway analysis for these populations showed enriched extracellular matrix organization, protein folding, positive regulation of cell adhesion, and collagen fibril formation (Supplementary Figure 4-1A-C). Collectively, I have shown that transcriptomic analysis of fibroblast subpopulations in the fibrotic lesions of experimental mice reveals specific fibroblast heterogeneity consistent with previously reported literature.


A
Col1a2-Cre(ER)-T mice (Denton et al., 2001)
Gt(ROSA)26mTmG mice (Muzumdar et al., 2007)
6kb proα2(I) collagen enhancer
Cre-ER(T)
IRES
hpAP
Poly A
R26R
tdT
Stop
GFP
loxP
loxP
Col1a2-Cre(ER)-T;Gt(ROSA)26mTmG mice (Tsang et al., 2019)
+ Tamoxifen
GFP expression in collagen-producing fibroblasts
tdTomato expression in all other cells
Inject subcutaneously with PBS or bleomycin ± scrambled peptide or BLR-200 for 10 days (as described in Section 4.2.1)
Fibroblast extraction from skin
Skin tissue collection for proteomic analysis
Fluorescence activated cell sorting to isolate collagen-expressing fibroblast population based on GFP expression
Single-cell RNA sequencing analysis

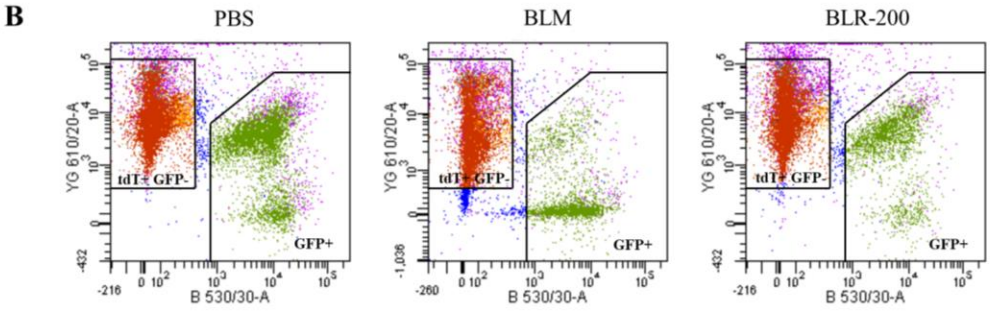
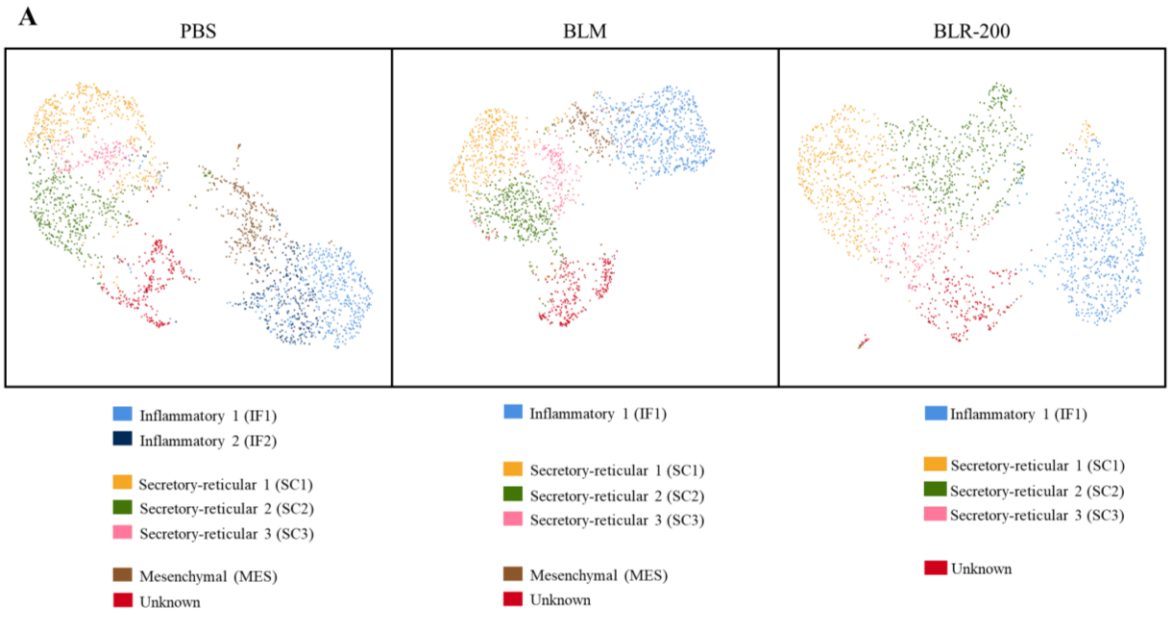
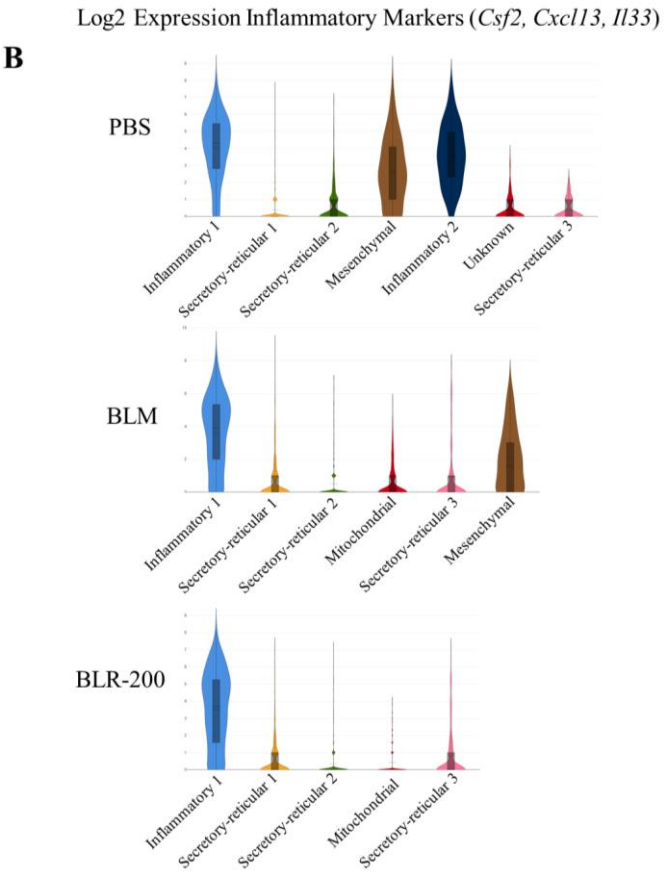

B
PBS
BLM
BLR-200
YG 610/20-A
B 530/30-A
tdT+ GFP-
GFP+

Figure 4-2. Isolation of collagen-lineage fibroblasts from the fibrotic lesion of mice subjected to bleomycin-induced dermal fibrosis.

(A) Illustration of workflow for labeling and isolation of collagen-lineage fibroblasts for scRNA-seq analysis. Collagen-lineage fibroblasts were labeled with GFP using a fibroblast specific collagen promoter/enhancer. Briefly, experimental mice were hemizygous for tamoxifen-dependent Cre recombinase under the control of the fibroblast specific proα2 collagen promoter (*Col1a2-Cre(ER)-T*; Denton et al., 2001) and homozygous for a double fluorescent reporter transgene (mT/mG) integrated into the *Gt(ROSA)26Sor* locus (*GT(ROSA)26mTmG*; Muzumdar et al., 2007). At 3 weeks of age, mice were injected with tamoxifen to induce GFP expression. After two weeks, bleomycin sulfate (0.1 units/100 µl per injection) or PBS (100 µl per injection) was injected subcutaneously into a single location on the flank of the mice once daily for 10 days. Bleomycin-treated mice were further divided into two treatment groups, which were injected intraperitoneally 3 times per week with either 10 µg/kg scrambled peptide or 10 µg/kg BLR-200. At the conclusion of the experiment, dermal tissue from the fibrotic lesion was collected. Fibroblasts were extracted from the dermis and GFP-expressing cells were isolated by fluorescence activated cell sorting (FACS) before being subjected to scRNA-seq analysis. **(B)** Fibroblasts were isolated from the fibrotic lesions using FACS, based on their expression of GFP.


A
PBS
BLM
BLR-200
Inflammatory 1 (IF1)
Inflammatory 2 (IF2)
Secretory-reticular 1 (SC1)
Secretory-reticular 2 (SC2)
Secretory-reticular 3 (SC3)
Mesenchymal (MES)
Unknown
Inflammatory 1 (IF1)
Secretory-reticular 1 (SC1)
Secretory-reticular 2 (SC2)
Secretory-reticular 3 (SC3)
Mesenchymal (MES)
Unknown
Inflammatory 1 (IF1)
Secretory-reticular 1 (SC1)
Secretory-reticular 2 (SC2)
Secretory-reticular 3 (SC3)
Unknown


B
Log2 Expression Inflammatory Markers (Csf2, Cxcl13, Il33)
PBS
Inflammatory 1
Secretory-reticular 1
Secretory-reticular 2
Mesenchymal
Inflammatory 2
Unknown
Secretory-reticular 3
BLM
Inflammatory 1
Secretory-reticular 1
Secretory-reticular 2
Mitochondrial
Secretory-reticular 3
Mesenchymal
BLR-200
Inflammatory 1
Secretory-reticular 1
Secretory-reticular 2
Mitochondrial
Secretory-reticular 3

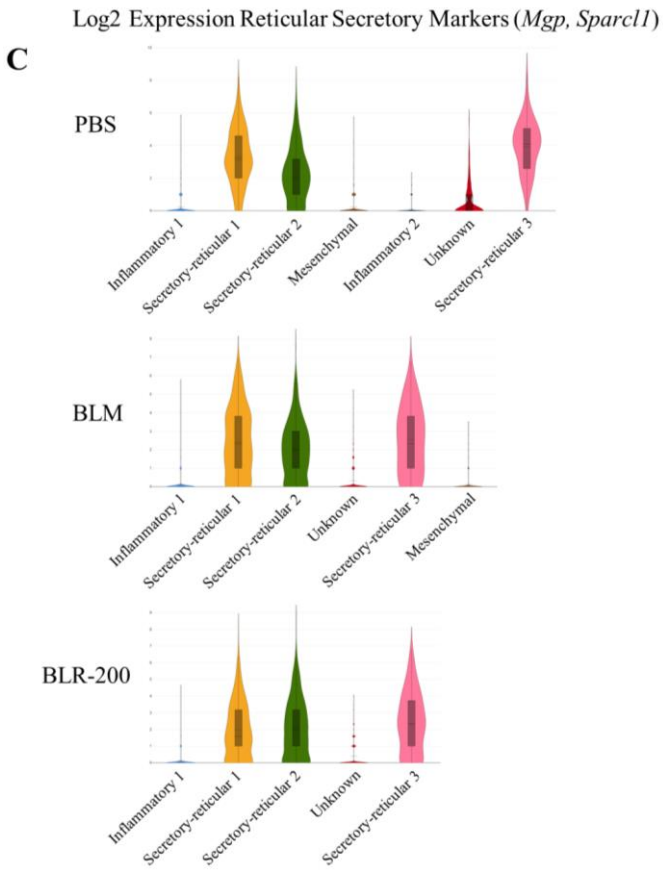
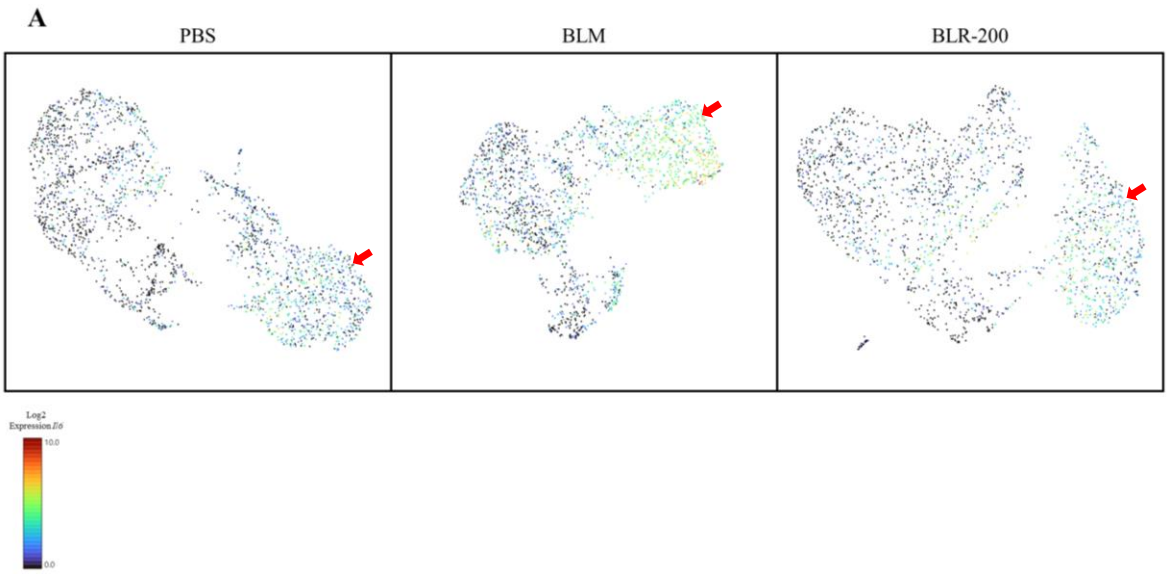


Figure 4-3. Identification of collagen-lineage fibroblast subpopulations in bleomycin-induced dermal fibrosis.

Mice were treated with either PBS (n=3), bleomycin + scrambled peptide (BLM; n=3) or bleomycin + BLR-200 (BLR-200; n=3) for 10 days. At the conclusion of the experiment, collagen-lineage fibroblasts were extracted and isolated by FACS. Isolated cells were subjected to scRNA-seq analysis and approximately 2000 cells from each treatment were sequenced. **(A)** Unsupervised UMAP clustering of cells in each treatment group yielding a total of 7 distinct clusters among the different treatments, comprising 2 inflammatory fibroblast clusters (IF1, IF2), 3 reticular-secretory fibroblast clusters (SC1, SC2, SC3), 1 mesenchymal fibroblast cluster (MES), and 1 unknown cluster. **(B)** Violin plots of inflammatory fibroblast marker genes for cell type identification. Inflammatory fibroblast markers include: *Cxcl13*, *Csf2*, *Il33*. **(C)** Violin plots of reticular-secretory fibroblast marker genes for cell type identification. Reticular-secretory fibroblast markers include: *Mgp* and *Sparcl1*.

4.3.3 BLR-200 treatment prevents the bleomycin-induced increase in *Il6* and *Cxcl2* expression in the inflammatory fibroblast subpopulation

I next wanted to assess transcriptomic changes occurring in the fibroblast subpopulations among treatment groups. It was clear from the UMAPs that there was a shift in the inflammatory fibroblast subpopulation (IF1) relative to the reticular secretory subpopulations (Figure 4-3A). Thus, I sought to determine the gene expression changes that were causing this shift. I looked at the list of most variable genes and identified that interleukin-6 (*Il6*) and *Cxcl2* were expressed at higher levels in the IF1 inflammatory subpopulation of BLM mice compared to PBS; however, this increase was impaired in the IF1 inflammatory subpopulation of BLR-200 mice (Figure 4-4A; 4-4B). IL-6 plays a pivotal role in driving acute inflammation and has also been shown to contribute to unresolved inflammation in fibrosis, including in SSc (Fielding et al., 2014; Johnson et al., 2020; Kawaguchi, 2017; Khan et al., 2012). CXCL2 is a chemokine that plays a role in many inflammatory processes and is also upregulated in the dermal fibroblasts of SSc patients (Johnson et al., 2015; Sahin & Wasmuth, 2013). Altogether, these data indicate that BLR-200 prevented pro-inflammatory transcriptomic changes in collagen-lineage dermal fibroblasts responding to bleomycin-induced dermal fibrosis.


A
PBS
BLM
BLR-200
Log2 Expression Il6
10.0
0.0

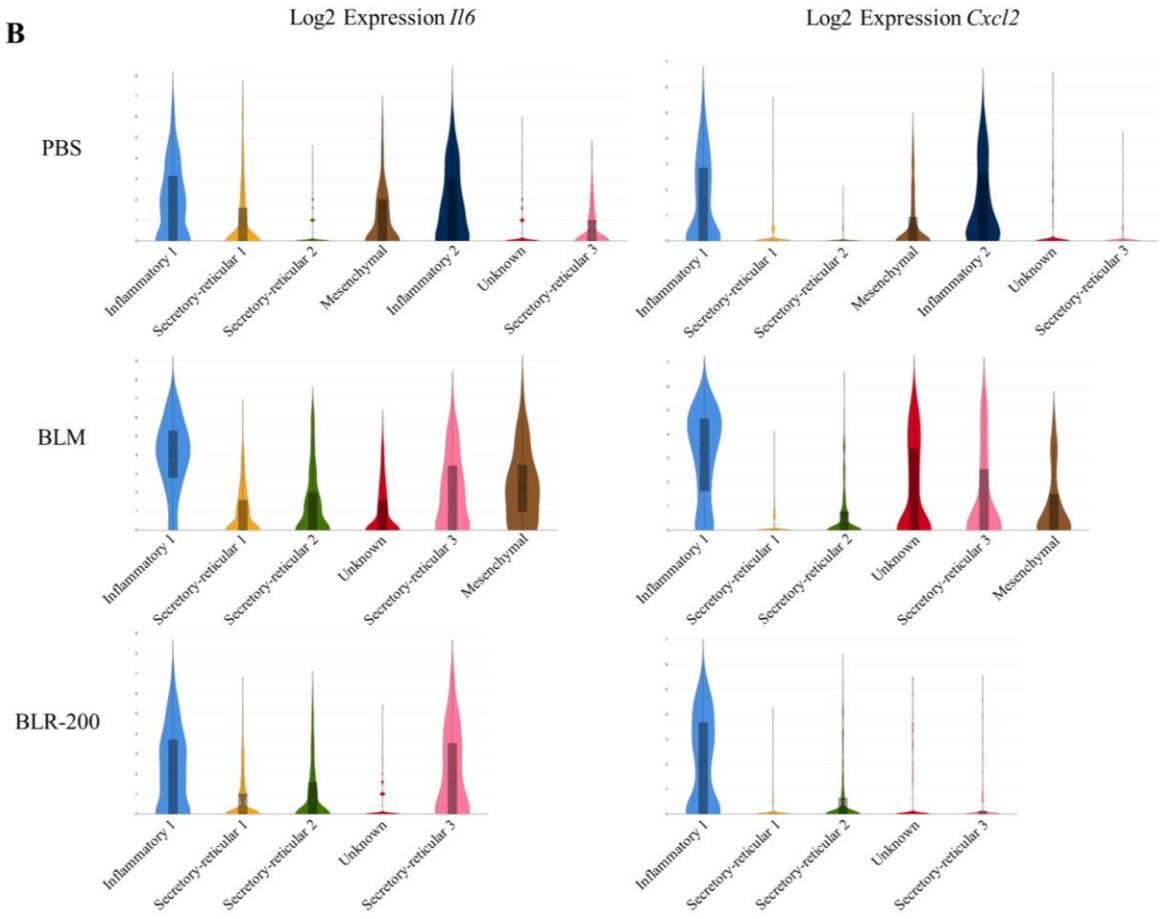
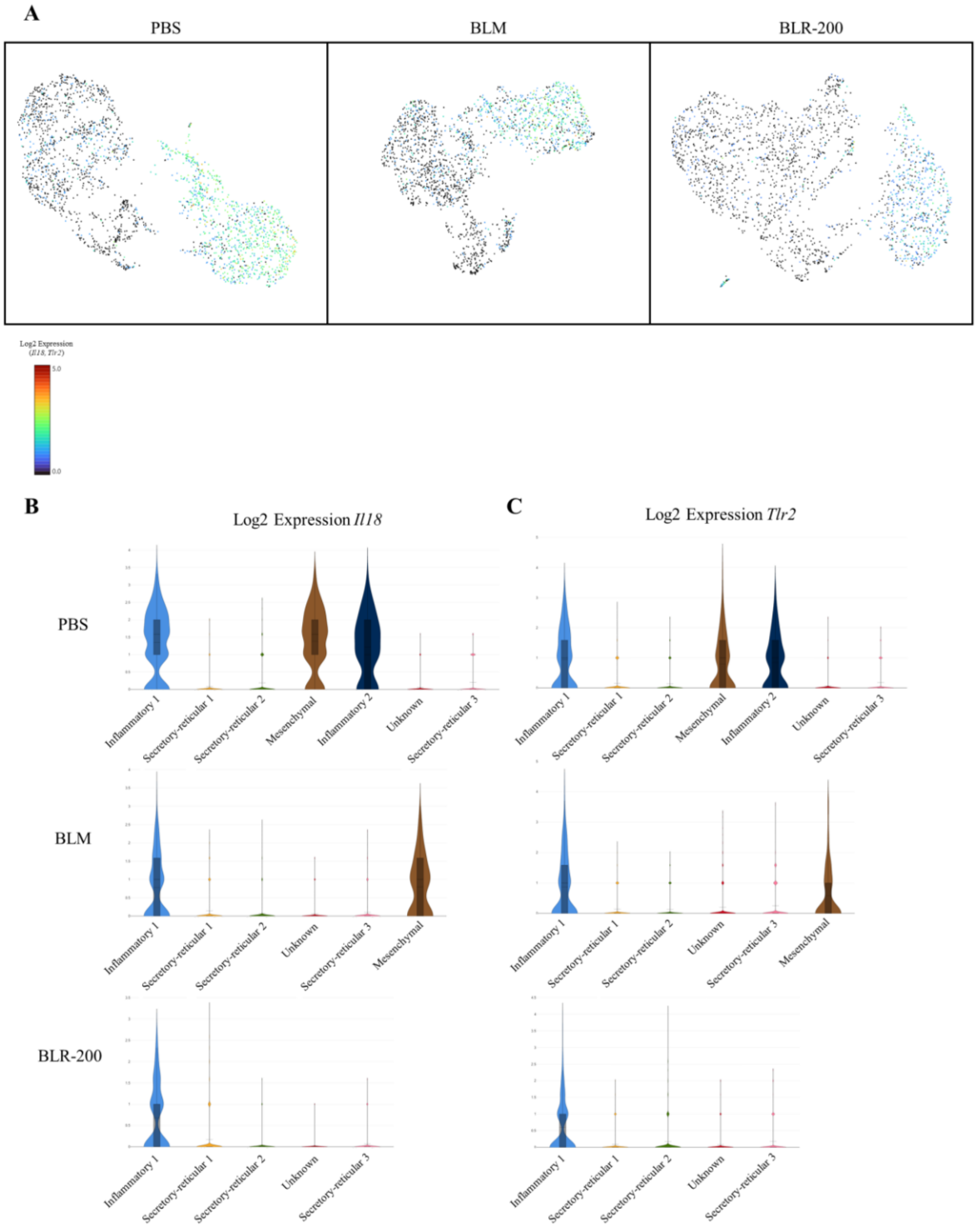

B
Log2 Expression Il6
Log2 Expression Cxcl2
PBS
BLM
BLR-200
Inflammatory 1
Secretory-reticular 1
Secretory-reticular 2
Mesenchymal
Inflammatory 2
Unknown
Secretory-reticular 3

Figure 4-4. BLR-200 prevents the bleomycin-induced transcriptomic changes in the inflammatory fibroblast subpopulation.

Mice were treated with either PBS (n=3), bleomycin + scrambled peptide (BLM; n=3) or bleomycin + BLR-200 (BLR-200; n=3) for 10 days. At the conclusion of the experiment, collagen-lineage fibroblasts were extracted and isolated by FACS. Isolated cells were subjected to scRNA-seq analysis and approximately 2000 cells from each treatment were sequenced. **(A)** Unsupervised UMAP clustering of cells reveals that BLM mice have a shift in the inflammatory subpopulation. The inflammatory subpopulation of BLM mice expresses higher levels of the pro-fibrotic, pro-inflammatory *Il6*. Cells are coloured based on Log2(*Il6* expression). **(B)** Violin plots showing Log2 expression of *Il6* and *Cxcl2*. BLR-200 prevents the bleomycin-induced increase in gene expression of *Il6* and *Cxcl2* in the inflammatory fibroblast subpopulation.

4.3.4 BLR-200 prevents expression of an NLRP3 inflammasome-related gene signature

To continue my assessment of BLR-200 on bleomycin-induced pro-inflammatory changes among fibroblast subpopulations, I analyzed the list of most variable genes for each cluster and used violin plots to compare expression of several genes of interest. As exhibited by UMAPs (Figure 4-5A) and violin plots, I found that BLR-200-treated mice had impaired expression of interleukin-18 (*Il18*), toll-like receptor 2 (*Tlr2*), interleukin-1 receptor type 1 (*Il1r1*), and interleukin-1 receptor type 2 (*Il1r2*) (Figure 4-5B; 4-5C; 4-5D; 4-5E). This impairment was most obvious in the IF1 inflammatory subpopulation. This genetic signature is associated with activation of the NLRP3 inflammasome, which has been implicated in fibrogenesis in the liver, kidney, and lung (Colak et al., 2021; Hsu et al., 2021; Rastrick & Birrell, 2014). Moreover, activation of the inflammasome also mediates the early innate immune response in patients with SSc, and an inflammasome-related gene signature is associated with a more aggressive fibroblast in SSc (Artlett et al., 2011; Henderson et al., 2018; Martinez-Godinez et al., 2015). These results provide further evidence that BLR-200 can impair the early, inflammatory changes of bleomycin-induced dermal fibrosis.


A
PBS
BLM
BLR-200
Log2 Expression
(Il18, Tlr2)
5.0
0.0
B
Log2 Expression Il18
PBS
BLM
BLR-200
Inflammatory 1
Secretory-reticular 1
Secretory-reticular 2
Mesenchymal
Inflammatory 2
Unknown
Secretory-reticular 3
C
Log2 Expression Tlr2
Inflammatory 1
Secretory-reticular 1
Secretory-reticular 2
Mesenchymal
Inflammatory 2
Unknown
Secretory-reticular 3

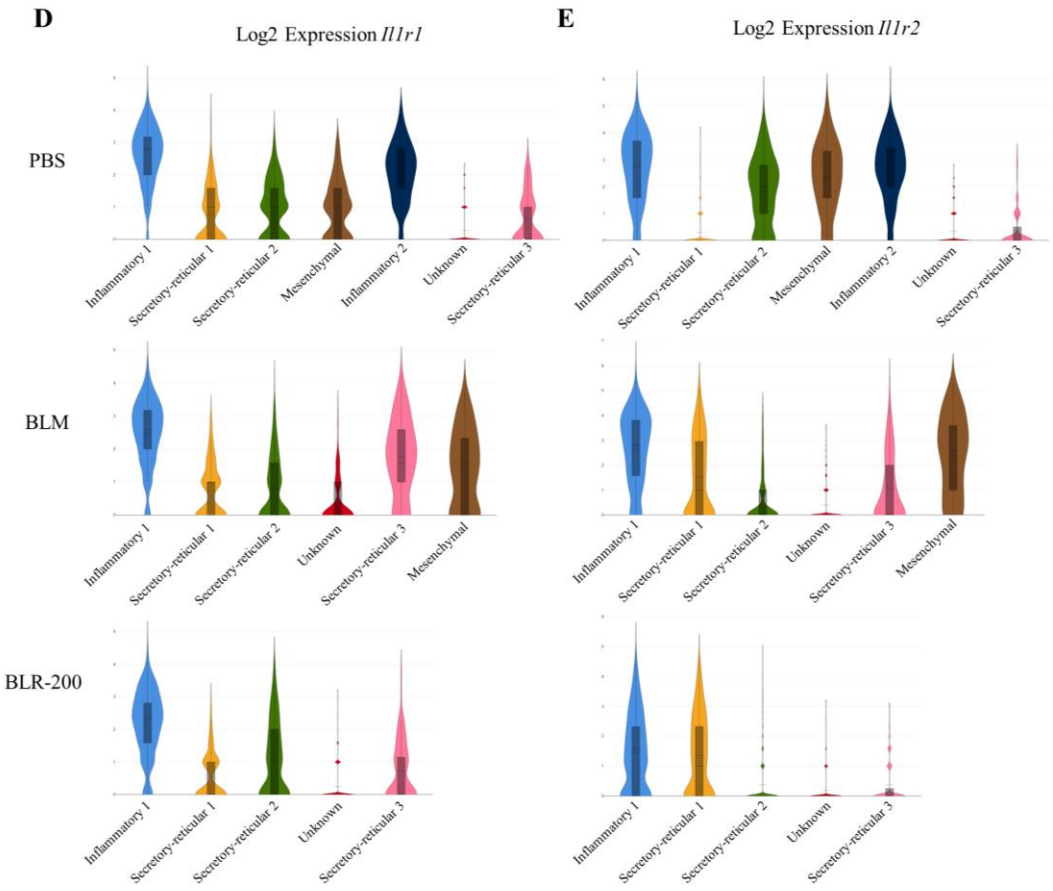

D
Log2 Expression Il1r1
E
Log2 Expression Il1r2
PBS
BLM
BLR-200
Inflammatory 1
Secretory-reticular 1
Secretory-reticular 2
Mesenchymal
Inflammatory 2
Unknown
Secretory-reticular 3

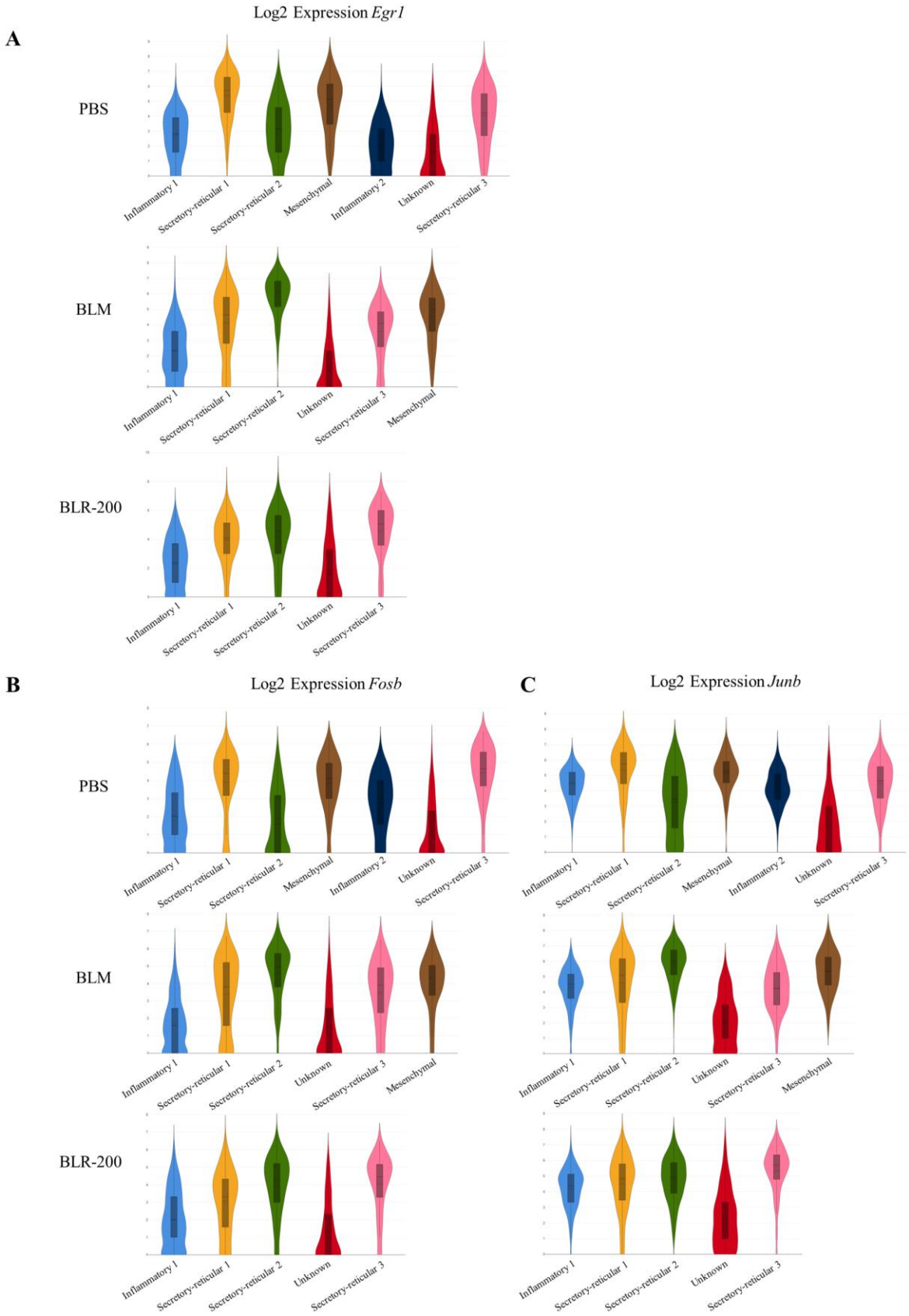
Figure 4-5. BLR-200 prevents expression of an NLRP3 inflammasome-related gene signature.

Mice were treated with either PBS (n=3), bleomycin + scrambled peptide (BLM; n=3) or bleomycin + BLR-200 (BLR-200; n=3) for 10 days. At the conclusion of the experiment, collagen-lineage fibroblasts were extracted and isolated by FACS. Isolated cells were subjected to scRNA-seq analysis and approximately 2000 cells from each treatment were sequenced. **(A)** Unsupervised UMAP clustering of cells reveals that expression of NLRP3 inflammasome markers *Il18* and *Tlr2* is lower in BLR-200 treated mice. Cells are coloured based on Log2(*Il18* and *Tlr2* expression). Violin plots also reveal that BLR-200 impairs expression of inflammasome-related markers **(B)** *Il18*, **(C)** *Tlr2*, **(D)** *Il1r1*, and **(E)** *Il1r2*.

4.3.5 BLR-200 specifically prevents the bleomycin-induced increase in the pro-fibrotic transcription factor *Egr1*

To further assess the ability of BLR-200 to prevent early, inflammatory changes in bleomycin-induced dermal fibrosis, I wanted to investigate transcriptomic changes in expression of pro-fibrotic transcription factors. Once again, I analyzed the list of most variable genes, and used violin plots to compare the expression of several genes of interest. In the secretory-reticular (SC2) fibroblast subpopulation of BLM mice, expression of early growth response-1 (*Egr1*) was increased; a change that was prevented by BLR-200 treatment (Figure 4-6A). EGR1, a multifunctional transcription factor, promotes fibroblast activation and fibrotic gene expression; an EGR1 gene expression signature is upregulated in a cohort of dcSSc patients (Bhattacharyya et al., 2013). EGR1 also mediates the early influx of inflammatory cells into fibrotic lesions and plays a role in regulating TGFβ activity in pathological matrix remodeling (Wu et al., 2009).

To assess whether BLR-200 specifically suppressed *Egr1* or more generally impaired expression of transcription factors known to promote fibrosis, I investigated if addition of BLR-200 also impeded the increased expression of members of the Activator Protein 1 (AP1) transcription factor complex (Avouac et al., 2012; Ponticos et al., 2009; Wernig et al., 2017). However, BLR-200 had minimal effects on bleomycin-induced increase in *Fosb* and *Junb* (Figure 4-6B). These results are consistent with the notion that BLR-200 selectively affects particular aspects of the fibroblast activation in response to inflammatory stimuli, and thus would appear to represent an extremely specific anti-fibrotic drug.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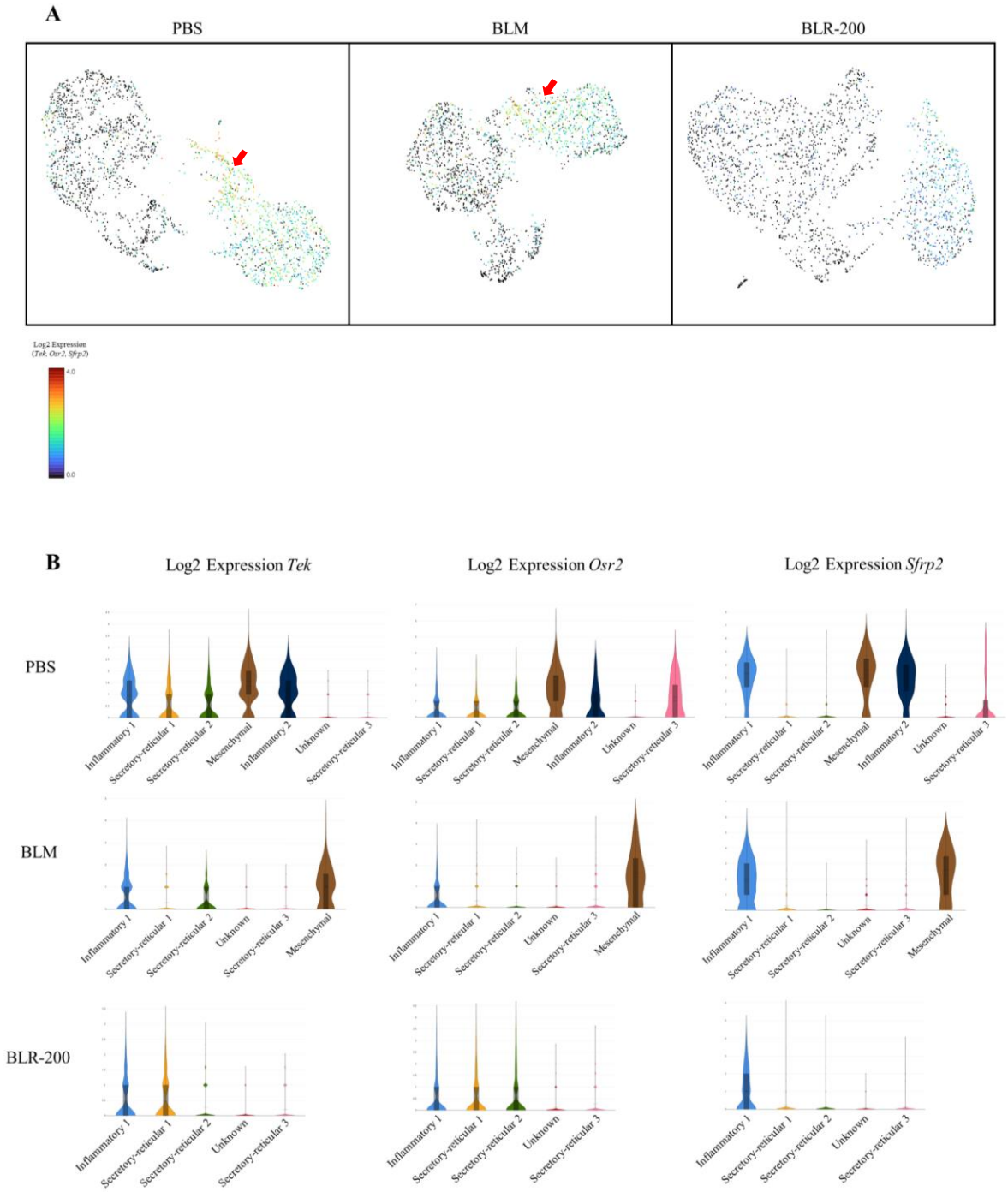
Log2 Expression Egr1
PBS
BLM
BLR-200
Inflammatory 1
Secretory-reticular 1
Secretory-reticular 2
Mesenchymal
Inflammatory 2
Unknown
Secretory-reticular 3
B
Log2 Expression Fosb
C
Log2 Expression Junb

Figure 4-6. BLR-200 has specific effects on pro-fibrotic transcription factors.

Mice were treated with either PBS (n=3), bleomycin + scrambled peptide (BLM; n=3) or bleomycin + BLR-200 (BLR-200; n=3) for 10 days. At the conclusion of the experiment, collagen-lineage fibroblasts were extracted and isolated by FACS. Isolated cells were subjected to scRNA-seq analysis and approximately 2000 cells from each treatment were sequenced. **(A)** Violin plot showing Log2 expression of *Egr1*. BLR-200 prevents the bleomycin-induced increase in gene expression of Egr-1 in the Secretory-reticular (SC2) subpopulation. Violin plots of Activator protein (AP1) transcription factor complex components, showing Log2 expression of **(B)** *Fosb* and **(C)** *Junb*.

4.3.6 BLR-200 prevents the appearance of a mesenchymal fibroblast subpopulation

After identifying multiple fibroblast subpopulations in each treatment group, I noticed that a mesenchymal fibroblast subpopulation (MES) was present in PBS and BLM mice, but not in BLR-200 treated mice. This mesenchymal subpopulation was characterized by expression of genes involved in development of the vasculature, skeletal system, and connective tissue (Ascensión et al., 2021; Deng et al., 2021; Solé-Boldo et al., 2020) (Supplementary Figure 4-1A-B). This subpopulation was confirmed in my study using UMAPs (Figure 4-7A) and violin plots (Figure 4-7B) looking at expression of angiopoietin-1 receptor (*Tek*), protein odd-skipped-related 2 (*Osr2*), and secreted frizzled-related protein 2 (*Sfrp2*), which are all involved in mesenchymal processes (Ascensión et al., 2021; Solé-Boldo et al., 2020). In BLR-200 mice, these markers did not appear at a significant level in any of the identified subpopulations. Additionally, this mesenchymal subpopulation expressed Wnt family member 10b (*Wnt10b*) and endothelin-1 (*Edn1*), both of which have been implicated in fibrogenesis in SSc (Chadli et al., 2019; Fonseca et al., 2006; Koçak et al., 2020) (Figure 4-7C; 4-7D). This is consistent with recent evidence suggesting that a mesenchymal fibroblast subpopulation is crucial in fibrotic skin diseases such as SSc (Deng et al., 2021). These results therefore indicate that BLR-200 treatment may prevent the formation of a potentially pro-fibrotic mesenchymal-like fibroblast subpopulation.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BLM
BLR-200
Log2 Expression
(Tek, Osr2, Sfrp2)
4.0
0.0
B
Log2 Expression Tek
Log2 Expression Osr2
Log2 Expression Sfrp2
PBS
BLM
BLR-200
Inflammatory 1
Secretory-reticular 1
Secretory-reticular 2
Mesenchymal
Inflammatory 2
Unknown
Secretory-reticular 3

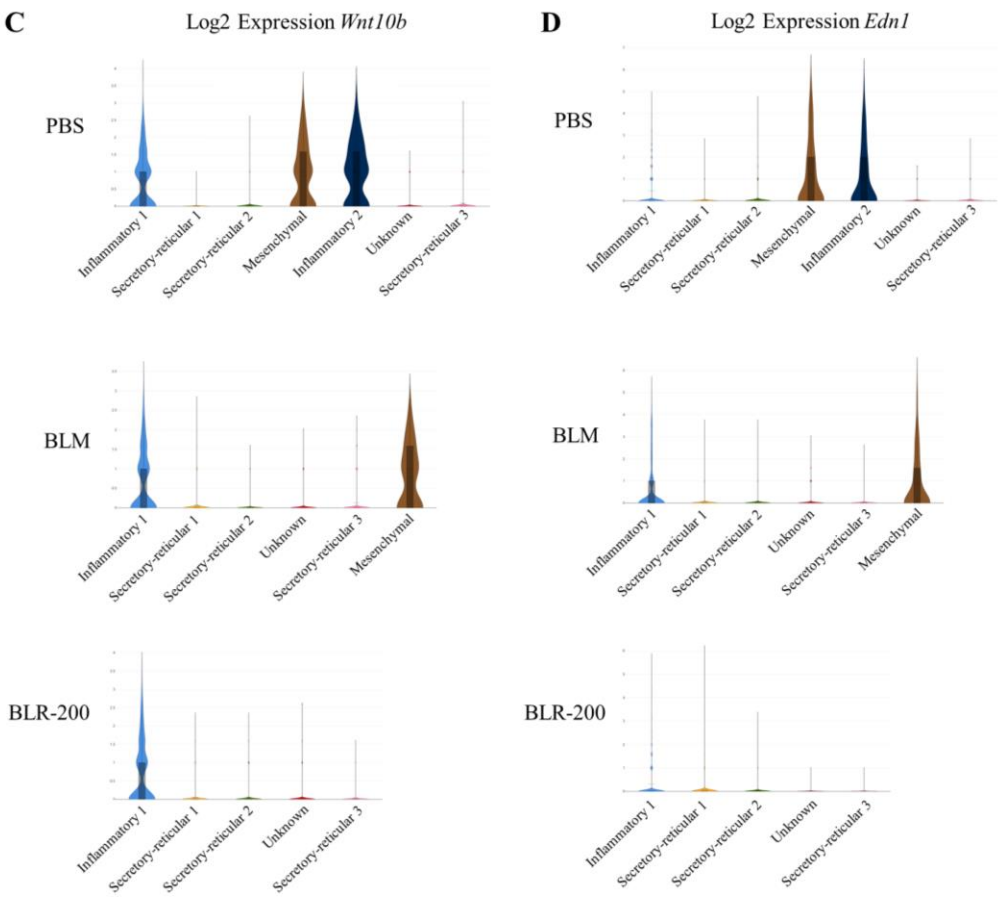


Figure 4-7. BLR-200 prevents the appearance of a mesenchymal fibroblast subpopulations.

Mice were treated with either PBS (n=3), bleomycin + scrambled peptide (BLM; n=3) or bleomycin + BLR-200 (BLR-200; n=3) for 10 days. At the conclusion of the experiment, collagen-lineage fibroblasts were extracted and isolated by FACS. Isolated cells were subjected to scRNA-seq analysis and approximately 2000 cells from each treatment were sequenced. **(A)** Unsupervised UMAP clustering of cells reveals that expression of mesenchymal markers *Tek*, *Osr2*, and *Sfrp2* are not appreciably present in BLR-200 treated mice. Cells are coloured based on Log2 expression of *Tek*, *Osr2*, and *Sfrp2*. **(B)** Violin plots showing Log2 expression of *Tek*, *Osr2* and *Sfrp2*. BLR-200 treated mice do not appreciably express these mesenchymal markers in any fibroblast subpopulations. Violin plots showing Log2 expression of **(C)** *Wnt10b* and **(D)** *Edn1*. BLR-200 treatment prevents expression of these pro-fibrotic genes.

4.4 Discussion

Currently, there is no approved therapy for treating the fibrosis observed in SSc. This deficiency can be attributed to multiple factors, including: (a) the etiology of SSc is relatively unknown; (b) knowledge gaps in the understanding of fibrogenesis; and (c) a lack of well-defined *in vivo* animal models to test potential anti-fibrotic therapies. The CCN family of matricellular proteins has emerged as an important player in fibrogenesis (Leask, 2020). CCN1 and CCN2, the most-studied members of the family, are upregulated in the connective tissue of early-onset SSc patients, and have pro-fibrotic effects (Sonnylal et al., 2010). CCN1 plays a role in collagen organization and has pro-inflammatory activity (Lau, 2011; Quesnel et al., 2019). CCN2 promotes a pro-fibrotic microenvironment by contributing to the persistent activation of myofibroblasts (Liu et al., 2011; Tsang et al., 2019). In contrast, CCN3 is downregulated in SSc (Xu Shi-wen and Richard Stratton, unpublished data), and has specific anti-fibrotic effects in kidney fibrosis (Riser et al., 2014). In this chapter, I show that targeting the regulation and activity of CCN proteins using BLR-200, a proprietary CCN3-derived peptide, can suppress specific early inflammatory and pro-fibrotic responses in the bleomycin-induced model of SSc dermal fibrosis. Furthermore, I use scRNA-seq to define the heterogenous collagen-lineage fibroblast subpopulation in the early inflammatory stage of bleomycin-induced fibrosis.

To investigate the early inflammatory stage of bleomycin-induced fibrosis, I analyzed tissue obtained 10 days post-initiation of bleomycin injection. This stage of the model precedes major fibrotic changes in the microenvironment, such as excessive collagen deposition (Yamamoto & Katayama, 2011). Early pro-fibrotic changes, such as keratinocyte and myofibroblast activation usually occur at this stage of fibrogenesis (Gabbiani, 2003; McCoy et al., 2017). My proteomic analysis revealed that BLR-200 treatment prevented these early pro-fibrotic changes. Notably, I found that markers of keratinocyte activation (keratin 1, 14, 15, 6) were prevented by BLR-200 treatment. These keratins are upregulated in the epidermis of fibrotic lesions in SSc patients (Aden et al., 2008; Nikitorowicz-Buniak et al., 2014). Previous studies have also shown that activated SSc keratinocytes likely play a role in early myofibroblast activation, through release of pro-fibrotic cytokines that can prime fibroblasts to become activated and to be hyper-

reactive to fibrotic stimuli (Aden et al., 2010; McCoy et al., 2017). Thus, impairing keratinocyte activation is an important anti-fibrotic effect elicited by BLR-200. Protein expression of other markers of early fibrogenesis and myofibroblast activation were also prevented by BLR-200, including fibrillin-1, dermatopontin, myosin light chain 6b, and tropomyosin 3 (Fujimura et al., 2011; Kissin et al., 2002; Malmstrom et al., 2004; Okamoto & Fujiwara, 2009). Collectively, these data indicate that BLR-200 can inhibit the increased inflammation-driven pro-fibrotic protein expression observed 10 days post-initiation of bleomycin injection.

Given the fact that, in SSc, keratinocyte activation precedes fibrotic changes in the microenvironment and promotes myofibroblast differentiation (Aden et al., 2010; McCoy et al., 2017), it is likely that fibroblasts within the lesional dermis are just starting to become activated 10 days post-initiation of bleomycin injection. Investigation of the transcriptomic changes occurring in fibroblasts at this point, and how they are affected by BLR-200, would therefore provide valuable information on potential mechanisms underlying fibrogenesis.

Thus, I employed unbiased scRNA-seq analysis to investigate the specific effects of BLR-200 on the heterogenous myofibroblast population and how it responds to early inflammatory changes in bleomycin-induced dermal fibrosis. Several recent studies have used scRNA-seq to characterize major fibroblast subpopulations in the dermis (Deng et al., 2021; Solé-Boldo et al., 2020). Solé-Boldo and colleagues sought to decipher fibroblast heterogeneity in aging human skin. The authors isolated and sequenced total cells from full-thickness skin samples. In young, healthy skin, they identified a fibroblast population that could be subdivided into four major subpopulations showing differential functional annotations. These subpopulations include an inflammatory subset, a secretory-reticular subset, a secretory-papillary subset, and a mesenchymal subset (Solé-Boldo et al., 2020). In a similar study, Deng and colleagues confirmed the presence of these four main subpopulations in human keloid scars (Deng et al., 2021). One caveat is that neither of these studies examined the role of these fibroblast subpopulations in active fibrosis. Furthermore, they were unable to make conclusions on the origin of these fibroblast subpopulations. Thus, their contributions to fibrogenesis remain to be determined. My

study contributes to this gap in knowledge. I used a GFP reporter construct to postnatally label resident *Col1a2*-expressing dermal fibroblasts. This population has been shown to contribute to the activated myofibroblast population in bleomycin-induced fibrosis (Liu et al., 2014; Tsang et al., 2019). Labelling these "pre-myofibroblasts" before they become activated provides distinct advantages in studying cellular changes in the early stages of fibrogenesis. My scRNA-seq analysis revealed that, in a murine model of dermal fibrosis, collagen-expressing dermal fibroblasts can give rise to fibroblast subpopulations that closely resemble previously identified inflammatory, secretory-reticular, and mesenchymal subpopulations. Notably, I did not observe a secretory-papillary subpopulation. This was not unexpected, as collagen-lineage myofibroblasts, which actively contribute to fibrosis, are not generally associated with the papillary dermis (Driskell & Watt, 2015).

To identify the fibroblast subpopulations in my experiment, I used functional pathway analysis, which provides a consistent guideline for functional annotation and subsequent fibroblast identification. In this report, the inflammatory subpopulations were enriched for pathways involved in cytokine- and chemokine-mediated signaling, neutrophil chemotaxis, positive regulation of chemokine production, and positive regulation of prostaglandin secretion. The secretory-reticular fibroblast subpopulations were enriched for pathways involved in extracellular matrix organization, protein folding, positive regulation of cell adhesion, and collagen fibril formation. Finally, the mesenchymal fibroblast subpopulations were enriched for pathways involved in skeletal system development, regulation of vasculature development, connective tissue development and cartilage development. I also used expression of known markers for inflammatory (*Csf2, Cxcl13, Il-33*), secretory-reticular (*Mgp, Sparcl1*) and mesenchymal (*Tek, Osr2, Sfrp2*) fibroblasts to provide further confirmation for this classification (Deng et al., 2021; Philippeos et al., 2018; Valenzi et al., 2019; Vorstandlechner et al., 2020). Although these markers differ slightly from previous scRNA-seq studies, this is likely due to species differences and general differences in experimental approach. Collectively, these data builds upon previous experiments investigating fibroblast heterogeneity (Deng et al., 2021; Solé-Boldo et al., 2020) and extends their classification of fibroblast subpopulations into a murine model of dermal fibrosis. Furthermore, investigation of BLR-200's specific effect on these fibroblast

subpopulations will allow us to determine the exact contribution of these cell types to dermal fibrosis.

One of the most obvious changes observed in my scRNA-seq data was the bleomycin-induced shift in the inflammatory fibroblast subpopulation (IF1), which was at least partially prevented by BLR-200. I found that a bleomycin-induced *Il-6/Cxcl2* expression pattern was contributing to this shift in the inflammatory subpopulation. IL-6 is a known pro-fibrotic cytokine and has long been recognized as an early marker of dermal fibrosis in SSc (Feghali et al., 1992; Hasegawa et al., 1998). Previous studies have also shown that prevention of IL-6 signaling attenuates bleomycin-induced dermal fibrosis (Kitaba et al., 2012). CXCL2 has been shown to play a role in multiple forms of fibrosis and is also upregulated in SSc (Johnson et al., 2015; King et al., 2018; Sahin & Wasmuth, 2013). Thus, impairment of these pro-fibrotic cytokines represents an important early step in prevention of fibrogenesis. Collectively, these data are consistent with the hypothesis that BLR-200 can prevent early pro-inflammatory changes in bleomycin-induced fibrosis.

Previous experiments have revealed the critical involvement of the NLRP3 inflammasome in promoting the early inflammatory events that result in fibrosis (Colak et al., 2021; Hsu et al., 2021; Martinez-Godinez et al., 2015). The NLRP3 inflammasome is a group of multimeric protein complexes involved in the innate immune response (Kelley et al., 2019). Toll-like-receptors (TLRs) play a role in activating intracellular NLRP3 inflammasome proteins, which results in activation and secretion of IL-18 and IL-1β (Henderson et al., 2018). In SSc, NLRP3 inflammasome-related genes are overexpressed, including *IL18*, *IL1β*, and *TLR*s (Broen et al., 2012; Martinez-Godinez et al., 2015). Moreover, NLRP3 inflammasome activation has been shown to contribute to myofibroblast activation and collagen secretion in fibroblasts (Artlett et al., 2011, 2017). Artlett and colleagues also showed that the NLRP3 inflammasome plays a crucial role in development of bleomycin-induced dermal fibrosis (Artlett et al., 2011). My scRNA-seq data reveals that BLR-200 impaired expression of NLRP3 inflammasome markers including *Il18, Tlr2, Il1r1,* and *Il1r2*. Given that activation of the inflammasome is a crucial early inflammatory change in fibrosis, this further emphasizes the ability of BLR-200 to impair fibrogenesis. To date, no studies have examined the direct relationship between CCN proteins and inflammasome

activity in pathological fibrosis. Therefore, it is not known whether the effects of BLR-200 on inflammasome activation are direct or indirect. However, CCN2 and inflammasome-related genes have been shown to be co-expressed in cardiac fibrosis, suggesting a potential relationship (Chandra et al., 2021).

Upon further assessment of scRNA-seq data, I found that BLR-200 also prevents the bleomycin-induced increase in *Egr1* expression. This was most apparent in the secretory-reticular fibroblast subpopulation (SC2). EGR1 is a transcription factor that responds to cellular stress, integrating extracellular signals and orchestrating early-immediate cellular responses to injury (Bhattacharyya, Wu, et al., 2011). EGR1 is overexpressed in the fibrotic tissue of SSc patients and induces an "EGR1 responsive gene signature" that is enriched in an inflammatory subset of dcSSc patients, thus likely plays a role in mediating the early inflammatory stage of fibrosis (Bhattacharyya, Sargent, et al., 2011). Moreover, Wu and colleagues showed that *Egr1*-null mice had an impaired inflammatory response and attenuated dermal fibrosis in response to bleomycin (Wu et al., 2009). I also investigated expression levels of other transcription factors known to be involved in fibrosis, including the AP1 transcription factor complex (Avouac et al., 2012). scRNA-seq analysis revealed increased expression of *Fosb* and *Junb* in response to bleomycin, however BLR had no observable effects on this change. Although unexpected, these results are consistent with the notion that BLR-200 selectively affects particular aspects of fibroblast activation, thus would appear to represent an extremely specific anti-fibrotic drug. The fact that EGR1 likely plays a role in mediating the early inflammatory stage of fibrogenesis is also significant in this case.

Another intriguing observation from my scRNA-seq data was the absence of the mesenchymal fibroblast subpopulation in BLR-200 treated mice. This subpopulation was characterized based on the expression of *Tek, Osr2*, and *Sfrp2*, and enrichment of functional pathways involved in development and differentiation (Deng et al., 2021; Solé-Boldo et al., 2020). The significance of this subpopulation in the context of fibrosis is not exactly clear. Although this mesenchymal subpopulation does not appear to display "traditional" pro-fibrotic expression patterns, it does express *Wnt10b* and *Edn1*, both of which have been implicated in SSc fibrogenesis (Fonseca et al., 2006; Koçak et al., 2020).

Therefore, this subpopulation may have slight pro-fibrotic activity. Furthermore, other scRNA-seq studies have reported that a mesenchymal fibroblast subpopulation is implicated in the fibrogenesis (Deng et al., 2021). Moreover, mesenchymal-like fibroblasts may be "primed" to induce a myofibroblast signature in response to the fibrotic microenvironment in SSc (Taki et al., 2020). Thus, the ability of BLR-200 to prevent this mesenchymal fibroblast subpopulation may have important implications on fibrogenesis in SSc. Future studies should be aimed at dissecting the significance of this subpopulation. Since this study was limited to a single timepoint, further studies aimed at investigating this mesenchymal subpopulation at multiple timepoints throughout fibrogenesis would be valuable.

In this chapter, I utilized unbiased scRNA-seq analysis to identify multiple fibroblast subpopulations in a model of dermal fibrosis that are consistent with previous observations (Ascensión et al., 2021; Solé-Boldo et al., 2020). These findings contribute to the overall understanding of fibroblast heterogeneity in bleomycin-induced dermal fibrosis. Furthermore, this study has built a base for further, more detailed analyses aimed at elucidating the exact roles of the diverse fibroblast populations in dermal fibrosis.

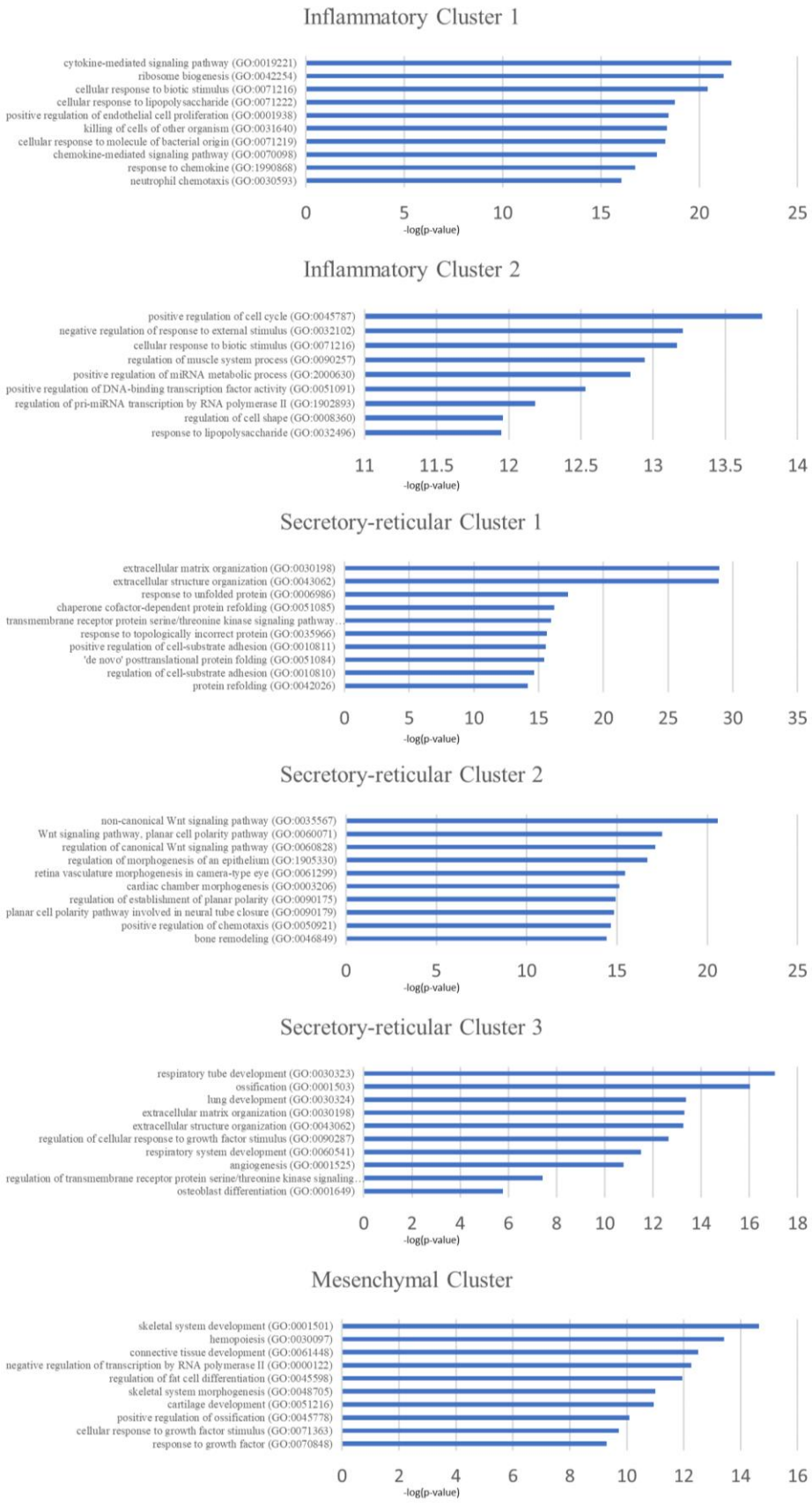
This study also reveals that that BLR-200, a CCN3-derived peptide, effectively prevented early pro-inflammatory changes in collagen-lineage fibroblasts and had specific inhibitory effects on the pro-fibrotic transcription factor EGR1. Preventing these changes likely impairs the formation of a pro-fibrotic microenvironment that permits activation and perpetuation of myofibroblasts. Thus, I have shown, for the first time, that CCN3 has anti-fibrotic effects, through impairment of the early inflammatory response in collagen-lineage fibroblasts, in an *in vivo* model of dermal fibrosis. This has widespread implications for the study of CCN proteins in fibrosis and further emphasizes the importance of studying this family of matricellular proteins in animal models.

A limitation of this study was the inability to complete immunohistological verification of the changes observed in my scRNA-seq analysis. This was due to the absence of reliable antibodies for the relevant markers, and limited tissue available for histology. Nevertheless, I have shown that BLR-200 prevents early pro-inflammatory and pro-fibrotic changes in a

murine model of SSc dermal fibrosis, emphasizing the therapeutic potential of using CCN3-based peptides to treat fibrosis.

4.5 Supplementary Figures

A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positive regulation of endothelial cell proliferation (GO:0001938)
killing of cells of other organism (GO:0031640)
cellular response to molecule of bacterial origin (GO:0071219)
chemokine-mediated signaling pathway (GO:0070098)
response to chemokine (GO:1990868)
neutrophil chemotaxis (GO:0030593)
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Inflammatory Cluster 2
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negative regulation of response to external stimulus (GO:0032102)
cellular response to biotic stimulus (GO:0071216)
regulation of muscle system process (GO:0090257)
positive regulation of miRNA metabolic process (GO:2000630)
positive regulation of DNA-binding transcription factor activity (GO:0051091)
regulation of pri-miRNA transcription by RNA polymerase II (GO:1902893)
regulation of cell shape (GO:0008360)
response to lipopolysaccharide (GO:0032496)
11 11.5 12 12.5 13 13.5 14
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extracellular structure organization (GO:0043062)
response to unfolded protein (GO:0006986)
chaperone cofactor-dependent protein refolding (GO:0051085)
transmembrane receptor protein serine/threonine kinase signaling pathway...
response to topologically incorrect protein (GO:0035966)
positive regulation of cell-substrate adhesion (GO:0010811)
'de novo' posttranslational protein folding (GO:0051084)
regulation of cell-substrate adhesion (GO:0010810)
protein refolding (GO:0042026)
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regulation of morphogenesis of an epithelium (GO:1905330)
retina vasculature morphogenesis in camera-type eye (GO:0061299)
cardiac chamber morphogenesis (GO:0003206)
regulation of establishment of planar polarity (GO:0090175)
planar cell polarity pathway involved in neural tube closure (GO:0090179)
positive regulation of chemotaxis (GO:0050921)
bone remodeling (GO:0046849)
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ossification (GO:0001503)
lung development (GO:0030324)
extracellular matrix organization (GO:0030198)
extracellular structure organization (GO:0043062)
regulation of cellular response to growth factor stimulus (GO:0090287)
respiratory system development (GO:0060541)
angiogenesis (GO:0001525)
regulation of transmembrane receptor protein serine/threonine kinase signaling...
osteoblast differentiation (GO:0001649)
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Mesenchymal Cluster
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hemopoiesis (GO:0030097)
connective tissue development (GO:0061448)
negative regulation of transcription by RNA polymerase II (GO:0000122)
regulation of fat cell differentiation (GO:0045598)
skeletal system morphogenesis (GO:0048705)
cartilage development (GO:0051216)
positive regulation of ossification (GO:0045778)
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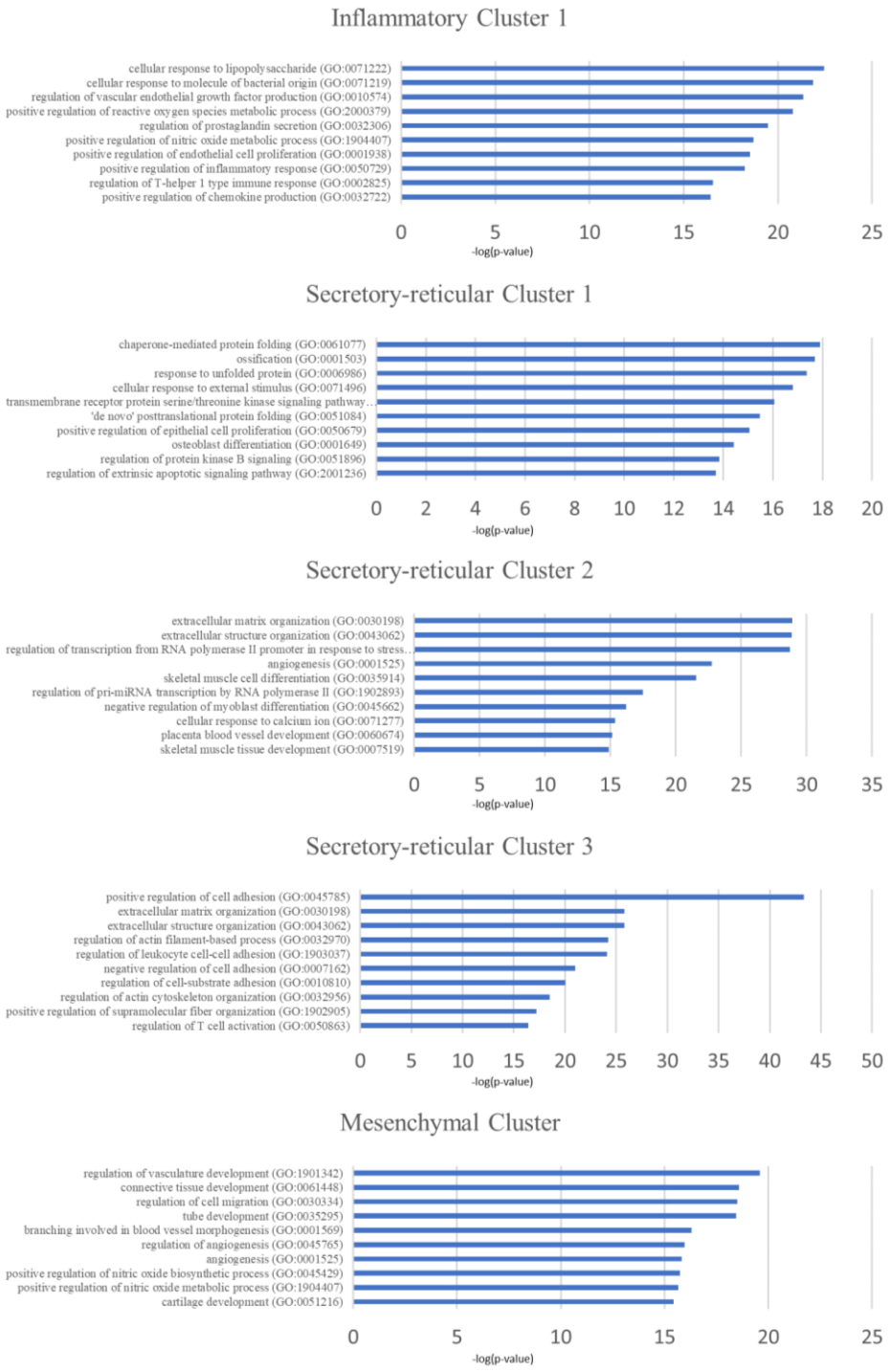
B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regulation of prostaglandin secretion (GO:0032306)
positive regulation of nitric oxide metabolic process (GO:1904407)
positive regulation of endothelial cell proliferation (GO:0001938)
positive regulation of inflammatory response (GO:0050729)
regulation of T-helper 1 type immune response (GO:0002825)
positive regulation of chemokine production (GO:0032722)
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Secretory-reticular Cluster 1
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ossification (GO:0001503)
response to unfolded protein (GO:0006986)
cellular response to external stimulus (GO:0071496)
transmembrane receptor protein serine/threonine kinase signaling pathway...
'de novo' posttranslational protein folding (GO:0051084)
positive regulation of epithelial cell proliferation (GO:0050679)
osteoblast differentiation (GO:0001649)
regulation of protein kinase B signaling (GO:0051896)
regulation of extrinsic apoptotic signaling pathway (GO:2001236)
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Secretory-reticular Cluster 2
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extracellular structure organization (GO:0043062)
regulation of transcription from RNA polymerase II promoter in response to stress...
angiogenesis (GO:0001525)
skeletal muscle cell differentiation (GO:0035914)
regulation of pri-miRNA transcription by RNA polymerase II (GO:1902893)
negative regulation of myoblast differentiation (GO:0045662)
cellular response to calcium ion (GO:0071277)
placenta blood vessel development (GO:0060674)
skeletal muscle tissue development (GO:0007519)
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-log(p-value)
Secretory-reticular Cluster 3
positive regulation of cell adhesion (GO:0045785)
extracellular matrix organization (GO:0030198)
extracellular structure organization (GO:0043062)
regulation of actin filament-based process (GO:0032970)
regulation of leukocyte cell-cell adhesion (GO:1903037)
negative regulation of cell adhesion (GO:0007162)
regulation of cell-substrate adhesion (GO:0010810)
regulation of actin cytoskeleton organization (GO:0032956)
positive regulation of supramolecular fiber organization (GO:1902905)
regulation of T cell activation (GO:0050863)
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Mesenchymal Cluster
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connective tissue development (GO:0061448)
regulation of cell migration (GO:0030334)
tube development (GO:0035295)
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regulation of angiogenesis (GO:0045765)
angiogenesis (GO:0001525)
positive regulation of nitric oxide biosynthetic process (GO:0045429)
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cartilage development (GO:0051216)
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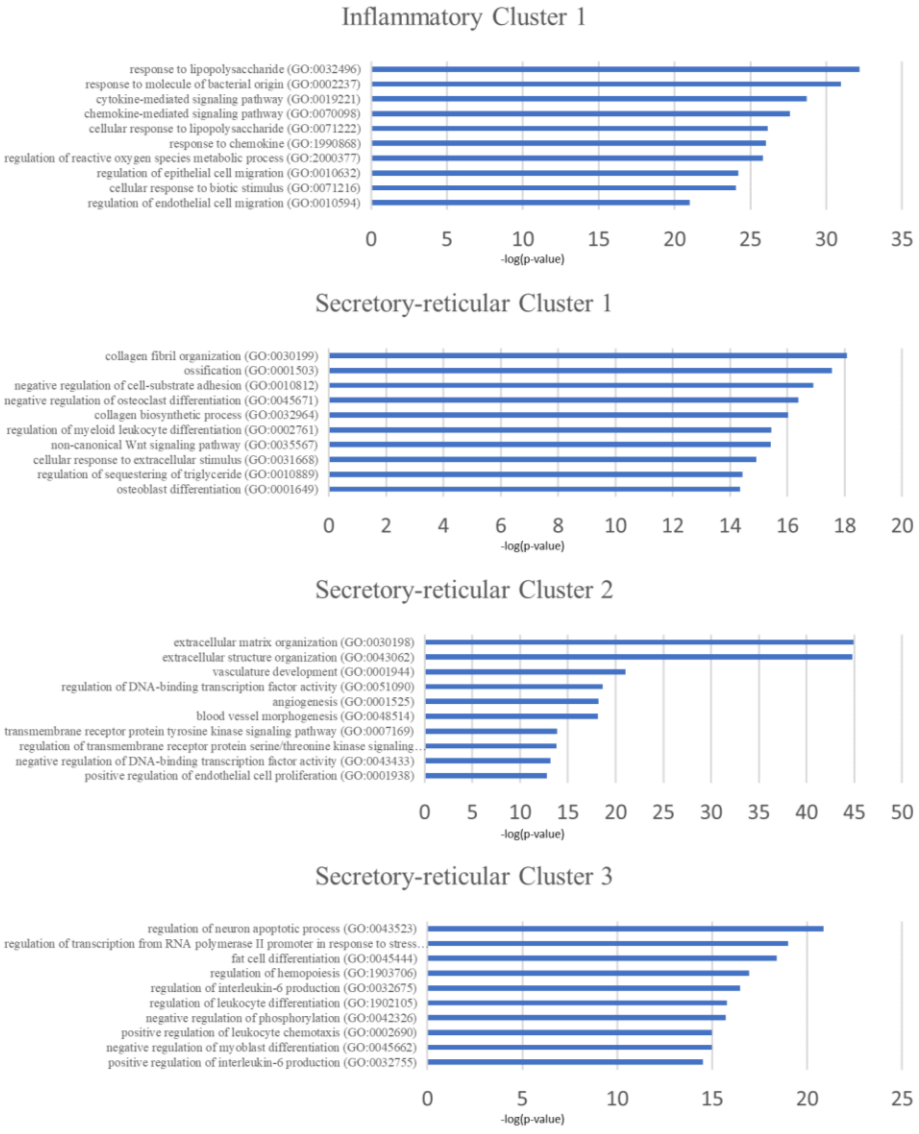
C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cytokine-mediated signaling pathway (GO:0019221)
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cellular response to lipopolysaccharide (GO:0071222)
response to chemokine (GO:1990868)
regulation of reactive oxygen species metabolic process (GO:2000377)
regulation of epithelial cell migration (GO:0010632)
cellular response to biotic stimulus (GO:0071216)
regulation of endothelial cell migration (GO:0010594)
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-log(p-value)
Secretory-reticular Cluster 1
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ossification (GO:0001503)
negative regulation of cell-substrate adhesion (GO:0010812)
negative regulation of osteoclast differentiation (GO:0045671)
collagen biosynthetic process (GO:0032964)
regulation of myeloid leukocyte differentiation (GO:0002761)
non-canonical Wnt signaling pathway (GO:0035567)
cellular response to extracellular stimulus (GO:0031668)
regulation of sequestering of triglyceride (GO:0010889)
osteoblast differentiation (GO:0001649)
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-log(p-value)
Secretory-reticular Cluster 2
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extracellular structure organization (GO:0043062)
vasculature development (GO:0001944)
regulation of DNA-binding transcription factor activity (GO:0051090)
angiogenesis (GO:0001525)
blood vessel morphogenesis (GO:0048514)
transmembrane receptor protein tyrosine kinase signaling pathway (GO:0007169)
regulation of transmembrane receptor protein serine/threonine kinase signaling...
negative regulation of DNA-binding transcription factor activity (GO:0043433)
positive regulation of endothelial cell proliferation (GO:0001938)
0 5 10 15 20 25 30 35 40 45 50
-log(p-value)
Secretory-reticular Cluster 3
regulation of neuron apoptotic process (GO:0043523)
regulation of transcription from RNA polymerase II promoter in response to stress...
fat cell differentiation (GO:0045444)
regulation of hemopoiesis (GO:1903706)
regulation of interleukin-6 production (GO:0032675)
regulation of leukocyte differentiation (GO:1902105)
negative regulation of phosphorylation (GO:0042326)
positive regulation of leukocyte chemotaxis (GO:0002690)
negative regulation of myoblast differentiation (GO:0045662)
positive regulation of interleukin-6 production (GO:0032755)
0 5 10 15 20 25
-log(p-value)

Figure 4-8, Supplementary Figure 1. Functional pathway assessment of fibroblast subpopulations identified in scRNA-seq analysis

Mice were treated with either PBS (n=3), bleomycin + scrambled peptide (BLM; n=3) or bleomycin + BLR-200 (BLR-200; n=3) for 10 days. At the conclusion of the experiment, collagen-lineage fibroblasts were extracted and isolated by FACS. Isolated cells were subjected to scRNA-seq analysis and approximately 2000 cells from each treatment were sequenced. Graphs showing the top enriched Gene Ontology (GO) terms in each fibroblast subpopulation, sorted by p-value. **(A)** PBS, **(B)** BLM, and **(C)** BLR-200.

4.6 References

Abd El Kader, T., Kubota, S., Janune, D., Nishida, T., Hattori, T., Aoyama, E., Perbal, B., Kuboki, T., & Takigawa, M. (2012). Anti-fibrotic effect of CCN3 accompanied by altered gene expression profile of the CCN family. *Journal of Cell Communication and Signaling*, *7*(1), 11–18. https://doi.org/10.1007/S12079-012-0180-4

Aden, N., Nuttall, A., Shiwen, X., Winter, P. de, Leask, A., Black, C. M., Denton, C. P., Abraham, D. J., & Stratton, R. J. (2010). Epithelial Cells Promote Fibroblast Activation via IL-1α in Systemic Sclerosis. *Journal of Investigative Dermatology*, *130*(9), 2191–2200. https://doi.org/10.1038/JID.2010.120

Aden, N., Shiwen, X., Aden, D., Black, C., Nuttall, A., Denton, C. P., Leask, A., Abraham, D., & Stratton, R. (2008). Proteomic analysis of scleroderma lesional skin reveals activated wound healing phenotype of epidermal cell layer. *Rheumatology*, *47*(12), 1754–1760. https://doi.org/10.1093/RHEUMATOLOGY/KEN370

Allanore, Y., Simms, R., Distler, O., Trojanowska, M., Pope, J., Denton, C. P., & Varga, J. (2015). Systemic sclerosis. *Nature Reviews Disease Primers*, *1*(15002), 1–21. https://doi.org/10.1038/nrdp.2015.2

Artlett, C. M., Sassi-Gaha, S., Hope, J. L., Feghali-Bostwick, C. A., & Katsikis, P. D. (2017). Mir-155 is overexpressed in systemic sclerosis fibroblasts and is required for NLRP3 inflammasome-mediated collagen synthesis during fibrosis. *Arthritis Research & Therapy*, *19*(1), 144. https://doi.org/10.1186/S13075-017-1331-Z

Artlett, C. M., Sassi-Gaha, S., Rieger, J. L., Boesteanu, A. C., Feghali-Bostwick, C. A., & Katsikis, P. D. (2011). The inflammasome activating caspase 1 mediates fibrosis and myofibroblast differentiation in systemic sclerosis. *Arthritis & Rheumatology*, *63*(11), 3563–3574. https://doi.org/10.1002/ART.30568

Ascensión, A. M., Fuertes-Álvarez, S., Ibañez-Solé, O., Izeta, A., & Araúzo-Bravo, M. J. (2021). Human Dermal Fibroblast Subpopulations Are Conserved across Single-Cell RNA Sequencing Studies. *Journal of Investigative Dermatology*, *141*(7), 1744. https://doi.org/10.1016/J.JID.2020.11.028

Avouac, J., Palumbo, K., Tomcik, M., Zerr, P., Dees, C., Horn, A., Maurer, B., Akhmetshina, A., Beyer, C., Sadowski, A., Schneider, H., Shiozawa, S., Distler, O., Schett, G., Allanore, Y., & Distler, J. H. W. (2012). Inhibition of activator protein 1 signaling abrogates transforming growth factor β–mediated activation of fibroblasts and prevents experimental fibrosis. *Arthritis & Rheumatology*, *64*(5), 1642–1652. https://doi.org/10.1002/ART.33501

Bhattacharyya, S., Fang, F., Tourtellotte, W., & Varga, J. (2013). Egr-1: new conductor for the tissue repair orchestra directs harmony (regeneration) or cacophony (fibrosis). *Journal of Pathology*, *229*(2), 286–297. https://doi.org/10.1002/path.4131

Bhattacharyya, S., Sargent, J. L., Lin, S., Tourtellotte, W. G., Takehara, K., Whitfield, M. L., & Varga, J. (2011). Egr-1 Induces a Profibrotic Injury/Repair Gene Program Associated with Systemic Sclerosis. *PLoS ONE*, *6*(9), e23082. https://doi.org/10.1371/journal.pone.0023082

Bhattacharyya, S., Wu, M., Fang, F., Tourtellotte, W., Feghali-Bostwick, C., & Varga, J. (2011). Early growth response transcription factors: key mediators of fibrosis and novel targets for anti-fibrotic therapy. *Matrix Biology*, *30*(4), 242. https://doi.org/10.1016/J.MATBIO.2011.03.005

Biernacka, A., Dobaczewski, M., & Frangogiannis, N. G. (2011). TGF-β signaling in fibrosis. *Growth Factors*. https://doi.org/10.3109/08977194.2011.595714

Bou-Gharios, G., Garrett, L. A., Rossert, J., Niederreither, K., Eberspaecher, H., Smith, C., Black, C., & Crombrugghe, B. (1996). A potent far-upstream enhancer in the mouse pro alpha 2(I) collagen gene regulates expression of reporter genes in transgenic mice. *Journal of Cell Biology*, *134*(5), 1333–1344. https://doi.org/10.1083/JCB.134.5.1333

Broen, J. C. A., Bossini-Castillo, L., van Bon, L., Vonk, M. C., Knaapen, H., Beretta, L., Rueda, B., Hesselstrand, R., Herrick, A., Worthington, J., Hunzelman, N., Denton, C. P., Fonseca, C., Riemekasten, G., Kiener, H. P., Scorza, R., Simeón, C. P., Ortego-Centeno, N., Gonzalez-Gay, M. A., … Radstake, T. R. D. J. (2012). A rare polymorphism in the gene for Toll-like receptor 2 is associated with systemic sclerosis phenotype and increases the production of inflammatory mediators. *Arthritis & Rheumatology*, *64*(1). https://doi.org/10.1002/art.33325

Bruce L. Riser. (2015). *CCN3 AND CCN3 PEPTIDES AND ANALOGS THEREOF FOR THERAPEUTC USES* (Patent No. US 9,114,112 B2).

Chadli, L., Sotthewes, B., Li, K., Andersen, S. N., Cahir-McFarland, E., Cheung, M., Cullen, P., Dorjée, A., de Vries-Bouwstra, J. K., Huizinga, T. W. J., Fischer, D. F., DeGroot, J., Viney, J. L., Zheng, T. S., Aarbiou, J., & Gardet, A. (2019). Identification of regulators of the myofibroblast phenotype of primary dermal fibroblasts from early diffuse systemic sclerosis patients. *Scientific Reports*, *9*(1), 1–12. https://doi.org/10.1038/s41598-019-41153-w

Chandra, S., Ehrlich, K. C., Lacey, M., Baribault, C., & Ehrlich, M. (2021). Epigenetics and expression of key genes associated with cardiac fibrosis: NLRP3, MMP2, MMP9, CCN2/CTGF and AGT. *Epigenomics*, *13*(3), 234. https://doi.org/10.2217/EPI-2020-0446

Chen, Y., Shi-wen, X., Beek, J. van, Kennedy, L., McLeod, M., Renzoni, E. A., Bou-Gharios, G., Wilcox-Adelman, S., Goetinck, P. F., Eastwood, M., Black, C. M., Abraham, D. J., & Leask, A. (2005). Matrix Contraction by Dermal Fibroblasts Requires Transforming Growth Factor-β/Activin-Linked Kinase 5, Heparan Sulfate-Containing Proteoglycans, and MEK/ERK: Insights into Pathological Scarring in Chronic Fibrotic Disease. *The American Journal of Pathology*, *167*(6), 1699. https://doi.org/10.1016/S0002-9440(10)61252-7

Colak, Y., Hasan, B., Erkalma, B., Tandon, K., Zervos, X., Menzo, E. lo, & Erim, T. (2021). Pathogenetic mechanisms of nonalcoholic fatty liver disease and inhibition of the inflammasome as a new therapeutic target. *Clinics and Research in Hepatology and Gastroenterology*, *45*(4), 101710. https://doi.org/10.1016/J.CLINRE.2021.101710

Coulombe, P. A. (1997). Towards a Molecular Definition of Keratinocyte Activation after Acute Injury to Stratified Epithelia. *Biochemical and Biophysical Research Communications*, *236*(2), 231–238. https://doi.org/10.1006/BBRC.1997.6945

Cutolo, M., Soldano, S., & Smith, V. (2019). Pathophysiology of systemic sclerosis: current understanding and new insights. *Expert Review of Clinical Immunology*, *15*(7), 753–764. https://doi.org/10.1080/1744666X.2019.1614915

Deng, C.-C., Hu, Y.-F., Zhu, D.-H., Cheng, Q., Gu, J.-J., Feng, Q.-L., Zhang, L.-X., Xu, Y.-P., Wang, D., Rong, Z., & Yang, B. (2021). Single-cell RNA-seq reveals fibroblast heterogeneity and increased mesenchymal fibroblasts in human fibrotic skin diseases. *Nature Communications*, *12*(3709), 1–16. https://doi.org/10.1038/s41467-021-24110-y

Denton, C. P., Wells, A. U., & Coghlan, J. G. (2018). Major lung complications of systemic sclerosis. *Nature Reviews Rheumatology*, *14*(9), 511–527. https://doi.org/10.1038/s41584-018-0062-0

Denton, C. P., Zheng, B., Shiwen, X., Zhang, Z., Bou-Gharios, G., Eberspaecher, H., Black, C. M., & de Crombrugghe, B. (2001). Activation of a Fibroblast-Specific Enhancer of the Pro2(I) Collagen Gene in Tight-Skin Mice. *Arthritis & Rheumatology*, *44*(3), 712–722. https://doi.org/10.1002/1529-0131

Desmoulière, A., Redard, M., Darby, I., & Gabbiani, G. (1995). Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *American Journal of Pathology*, *146*(1), 66. /pmc/articles/PMC1870783/?report=abstract

Driskell, R. R., & Watt, F. M. (2015). Understanding fibroblast heterogeneity in the skin. *Trends in Cell Biology*, *25*(2), 92–99. https://doi.org/10.1016/J.TCB.2014.10.001

Feghali, C., Bost, K., Boulware, D., & Levy, L. (1992). Mechanisms of pathogenesis in scleroderma. I. Overproduction of interleukin 6 by fibroblasts cultured from affected skin sites of patients with scleroderma. *Journal of Rheumatology*, *19*(8), 1207–1211. https://pubmed.ncbi.nlm.nih.gov/1404155/

Fielding, C. A., Jones, G. W., Mcloughlin, R. M., Mcleod, L., Hammond, V. J., Uceda, J., Williams, A. S., Lambie, M., Foster, T. L., Liao, C.-T., Rice, C. M., Greenhill, C. J., Colmont, C. S., Hams, E., Coles, B., Kift-Morgan, A., Newton, Z., Craig, K. J., Williams, J. D., … Jones, S. A. (2014). Article Interleukin-6 Signaling Drives Fibrosis in Unresolved Inflammation. *Immunity*, *40*, 40–50. https://doi.org/10.1016/j.immuni.2013.10.022

Fonseca, C., Renzoni, E., Sestini, P., Pantelidis, P., Lagan, A., Bunn, C., McHugh, N., Welsh, K. I., Bois, R. M. du, Denton, C. P., Black, C., & Abraham, D. (2006). Endothelin axis polymorphisms in patients with scleroderma. *Arthritis & Rheumatology*, *54*(9), 3034–3042. https://doi.org/10.1002/ART.22036

Fujimura, T., Hotta, M., Kitahara, T., & Takema, Y. (2011). Loss of contraction force in dermal fibroblasts with aging due to decreases in myosin light chain phosphorylation enzymes. *Archives of Pharmacal Research*, *34*(6), 1015–1022. https://doi.org/10.1007/S12272-011-0619-9

Gabbiani, G. (2003). The myofibroblast in wound healing and fibrocontractive diseases. In *Journal of Pathology*. https://doi.org/10.1002/path.1427

Hasegawa, M., Sato, S., Fujimoto, M., Ihn, H., Kikuchi, K., & Takehara, K. (1998). Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. *Journal of Rheumatology*, *25*(2), 308–313. https://doi.org/10.1080/14397595.2018.1559909

Henderson, J., Bhattacharyya, S., Varga, J., & O'Reilly, S. (2018). Targeting TLRs and the inflammasome in systemic sclerosis. *Pharmacology & Therapeutics*, *192*, 163–169. https://doi.org/10.1016/J.PHARMTHERA.2018.08.003

Hinz, B. (2010). The myofibroblast: Paradigm for a mechanically active cell. *Journal of Biomechanics*. https://doi.org/10.1016/j.jbiomech.2009.09.020

Hinz, B. (2015). Myofibroblasts. In *Experimental Eye Research*. https://doi.org/10.1016/j.exer.2015.07.009

Holmes, A. M., Ponticos, M., Shi-wen, X., Denton, C. P., & Abraham, D. J. (2011). Elevated CCN2 expression in scleroderma: a putative role for the TGFβ accessory receptors TGFβRIII and endoglin. *Journal of Cell Communication and Signaling*, *5*(3), 177. https://doi.org/10.1007/S12079-011-0140-4

Hsu, Y.-H., Zheng, C.-M., Chou, C.-L., Chen, Y.-J., Lee, Y.-H., Lin, Y.-F., Chiu, H.-W., & Therapeutic, H. (2021). Therapeutic Effect of Endothelin-Converting Enzyme Inhibitor on Chronic Kidney Disease through the Inhibition of Endoplasmic Reticulum Stress and the NLRP3 Inflammasome. *Biomedicines*, *9*(398), 1–14. https://doi.org/10.3390/biomedicines9040398

Johnson, B. Z., Stevenson, A. W., Prêle, C. M., Fear, M. W., & Wood, F. M. (2020). The Role of IL-6 in Skin Fibrosis and Cutaneous Wound Healing. *Biomedicines*, *8*(101), 1–18. https://doi.org/10.3390/biomedicines8050101

Johnson, M. E., Mahoney, J. M., Taroni, J., Sargent, J. L., Marmarelis, E., Wu, M.-R., Varga, J., Hinchcliff, M. E., & Whitfield, M. L. (2015). Experimentally-Derived Fibroblast Gene Signatures Identify Molecular Pathways Associated with Distinct Subsets of Systemic Sclerosis Patients in Three Independent Cohorts. *PLoS ONE*, *10*(1), e0114017. https://doi.org/10.1371/JOURNAL.PONE.0114017

Kawaguchi, Y. (2017). Contribution of Interleukin-6 to the Pathogenesis of Systemic Sclerosis. *Journal of Scleroderma and Related Disorders*, *2*, S6–S12. https://doi.org/10.5301/JSRD.5000258

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *International Journal of Molecular Sciences*, *20*(13), 3328. https://doi.org/10.3390/IJMS20133328

Khan, K., Xu, S., Nihtyanova, S., Derrett-Smith, E., Abraham, D., Denton, C. P., & Ong, V. H. (2012). Clinical and pathological significance of interleukin 6 overexpression in systemic sclerosis. *Annals of the Rheumatic Diseases*, *71*(7), 1235–1242. https://doi.org/10.1136/ANNRHEUMDIS-2011-200955

King, J., Abraham, D., & Stratton, R. (2018). Chemokines in systemic sclerosis. *Immunology Letters*, *195*, 68–75. https://doi.org/10.1016/J.IMLET.2017.12.001

Kissin, E. Y., Lemaire, R., Korn, J. H., & Lafyatis, R. (2002). Transforming growth factor β induces fibroblast fibrillin-1 matrix formation. *Arthritis & Rheumatology*, *46*(11), 3000–3009. https://doi.org/10.1002/ART.10621

Kitaba, S., Murota, H., Terao, M., Azukizawa, H., Terabe, F., Shima, Y., Fujimoto, M., Tanaka, T., Naka, T., Kishimoto, T., & Katayama, I. (2012). Blockade of Interleukin-6 Receptor Alleviates Disease in Mouse Model of Scleroderma. *American Journal of Pathology*, *180*(1), 165–176. https://doi.org/10.1016/J.AJPATH.2011.09.013

Koçak, A., Harmancı, D., Akdoğan, G. G., & Birlik, M. (2020). Relationship of Wnt pathway activity and organ involvement in scleroderma types. *International Journal of Rheumatic Diseases*, *23*(11), 1558–1567. https://doi.org/10.1111/1756-185X.13973

Lagares, D., Busnadiego, O., Ana García-Fernández, R., Kapoor, M., Liu, S., Carter, D. E., Abraham, D., Shi-Wen, X., Carreira, P., Fontaine, B. A., Shea, B. S., Tager, A. M., Leask, A., Lamas, S., & Rodríguez-Pascual, F. (2012). Inhibition of focal adhesion kinase prevents experimental lung fibrosis and myofibroblast formation. *Arthritis & Rheumatology*, *64*(5), 1653–1664. https://doi.org/10.1002/art.33482

Lane, E., & McLean, W. (2004). Keratins and skin disorders. *Journal of Pathology*, *204*(4), 355–366. https://doi.org/10.1002/PATH.1643

Lau, L. F. (2011). CCN1/CYR61: the very model of a modern matricellular protein. *Cellular and Molecular Life Sciences*, *68*(19). https://doi.org/10.1007/s00018-011-0778-3

Lau, L. F. (2016). Cell surface receptors for CCN proteins. *Journal of Cell Communication and Signaling*, *10*(2), 127. https://doi.org/10.1007/S12079-016-0324-Z

Leask, A. (2015). Matrix remodeling in systemic sclerosis. In *Seminars in Immunopathology*. https://doi.org/10.1007/s00281-015-0508-2

Leask, A. (2020a). The hard problem: Mechanotransduction perpetuates the myofibroblast phenotype in scleroderma fibrosis. *Wound Repair and Regeneration*, *29*, 582–587. https://onlinelibrary-wiley-com.proxy1.lib.uwo.ca/doi/pdfdirect/10.1111/wrr.12889

Leask, A. (2020b). A centralized communication network: Recent insights into the role of the cancer associated fibroblast in the development of drug resistance in tumors. *Seminars in Cell & Developmental Biology*, *101*, 111–114. https://doi.org/10.1016/J.SEMCDB.2019.10.016

Leask, A. (2020c). Conjunction junction, what's the function? CCN proteins as targets in fibrosis and cancers. *American Journal of Physiology - Cell Physiology*, *318*(6), C1046. https://doi.org/10.1152/AJPCELL.00028.2020

Lemaire, R., Farina, G., Bayle, J., Dimarzio, M., Pendergrass, S. A., Milano, A., Whitfield, M. L., & Lafyatis, R. (2010). Antagonistic Effect of the Matricellular Signaling

Protein CCN3 on TGF-β- and Wnt-Mediated Fibrillinogenesis in Systemic Sclerosis and Marfan Syndrome. *Journal of Investigative Dermatology*, *130*(6), 1514–1523. https://doi.org/10.1038/JID.2010.15

Liu, S., Herault, Y., Pavlovic, G., & Leask, A. (2014). Skin progenitor cells contribute to bleomycin-induced skin fibrosis. *Arthritis & Rheumatology*. https://doi.org/10.1002/art.38276

Liu, S., & Leask, A. (2013). CCN2 modulates hair follicle cycling in mice. *Molecular Biology of the Cell*, *24*(24), 3939–3944. https://doi.org/10.1091/MBC.E13-08-0472

Liu, S., Parapuram, S. K., & Leask, A. (2013). Fibrosis Caused by Loss of PTEN Expression in Mouse Fibroblasts Is Crucially Dependent on CCN2. *Arthritis & Rheumatology*, *65*(11), 2940–2944. https://doi.org/10.1002/ART.38121

Liu, S., Shi-Wen, X., Abraham, D. J., & Leask, A. (2011). CCN2 is required for bleomycin-induced skin fibrosis in mice. *Arthritis & Rheumatology*, *63*(1), 239–246. https://doi.org/10.1002/art.30074

Liu, S., Thompson, K., & Leask, A. (2014). CCN2 expression by fibroblasts is not required for cutaneous tissue repair. *Wound Repair and Regeneration*, *22*(1), 119–124. https://doi.org/10.1111/wrr.12131

Malmstrom, J., Linberg, H., Lindberg, C., Bratt, C., Wieslander, E., Delander, E. L., Särnstrand, B., Burns, J. S., Mose-Larsen, P., Fey, S., & Marko-Varga, G. (2004). Transforming Growth Factor-β1 Specifically Induce Proteins Involved in the Myofibroblast Contractile Apparatus. *Molecular & Cellular Proteomics*, *3*(5), 466–477. https://doi.org/10.1074/MCP.M300108-MCP200

Martinez-Godinez, M. A., Cruz-Dominguez, M. P., Jara, L. J., Dominguez-Lopez, A., Jarillo-Luna, R. A., Vera-Lastra, O., Montes-Cortes, D. H., Campos-Rodriguez, R., Lopez-Sanchez, D. M., Mejia-Barradas, C. M., Castelan-Chavez, E. E., & Miliar-Garcia, A. (2015). Expression of NLRP3 inflammasome, cytokines and vascular mediators in the skin of systemic sclerosis patients. *Israel Medical Association Journal*, *17*(1), 5–10. https://doi.org/10.1002/ART.30568

McCoy, S. S., Reed, T. J., Berthier, C. C., Tsou, P.-S., Liu, J., Gudjonsson, J. E., Khanna, D., & Kahlenberg, J. M. (2017). Scleroderma keratinocytes promote fibroblast activation independent of transforming growth factor β. *Rheumatology*, *56*(11), 1981. https://doi.org/10.1093/RHEUMATOLOGY/KEX280

Muangchan, C., & Pope, J. E. (2012). Interleukin 6 in Systemic Sclerosis and Potential Implications for Targeted Therapy. *Journal of Rheumatology*, *39*(6), 1120–1124. https://doi.org/10.3899/JRHEUM.111423

Muzumdar, M., Tasic, B., Miyamichi, K., Li, L., & Luo, L. (2007). A Global Double-Fluorescent Cre Reporter Mouse. *Genesis*, *45*, 593–605. https://doi.org/10.1002/dvg.20335

Nikitorowicz-Buniak, J., Shiwen, X., Denton, C. P., Abraham, D., & Stratton, R. (2014). Abnormally Differentiating Keratinocytes in the Epidermis of Systemic Sclerosis

Patients Show Enhanced Secretion of CCN2 and S100A9. *Journal of Investigative Dermatology*, *134*(11), 2693–2702. https://doi.org/10.1038/JID.2014.253

Okamoto, O., & Fujiwara, S. (2009). Dermatopontin, a Novel Player in the Biology of the Extracellular Matrix. *Connective Tissue Research*, *47*(4), 177–189. https://doi.org/10.1080/03008200600846564

Park, M.-H., Kim, A. kyung, Manandhar, S., Oh, S.-Y., Jang, G.-H., Kang, L., Lee, D.-W., Hyeon, D. Y., Lee, S.-H., Lee, H. E., Huh, T.-L., Suh, S. H., Hwang, D., Byun, K., Park, H.-C., & Lee, Y. M. (2019). CCN1 interlinks integrin and hippo pathway to autoregulate tip cell activity. *ELife*, *8*. https://doi.org/10.7554/ELIFE.46012

Perbal, B. (2018). The concept of the CCN protein family revisited: a centralized coordination network. *Journal of Cell Communication and Signaling*, *12*(1), 3. https://doi.org/10.1007/S12079-018-0455-5

Philippeos, C., Telerman, S. B., Oulès, B., Pisco, A. O., Shaw, T. J., Elgueta, R., Lombardi, G., Driskell, R. R., Soldin, M., Lynch, M. D., & Watt, F. M. (2018). Spatial and Single-Cell Transcriptional Profiling Identifies Functionally Distinct Human Dermal Fibroblast Subpopulations. *Journal of Investigative Dermatology*, *138*(4), 825. https://doi.org/10.1016/J.JID.2018.01.016

Ponticos, M., Abraham, D., Alexakis, C., Lu, Q. L., Black, C., Partridge, T., & Bou-Gharios, G. (2004). Col1a2 enhancer regulates collagen activity during development and in adult tissue repair. *Matrix Biology*, *22*(8), 619–628. https://doi.org/10.1016/J.MATBIO.2003.12.002

Ponticos, M., Harvey, C., Ikeda, T., Abraham, D., & Bou-Gharios, G. (2009). JunB mediates enhancer/promoter activity of COL1A2 following TGF-b induction. *Nucleic Acids Research*, *37*(16), 5378–5389. https://doi.org/10.1093/nar/gkp544

Quesnel, K., Shi-wen, X., Hutchenreuther, J., Xiao, Y., Liu, S., Peidl, A., Naskar, D., Siqueira, W. L., O'Gorman, D. B., Hinz, B., Stratton, R. J., & Leask, A. (2019). CCN1 expression by fibroblasts is required for bleomycin-induced skin fibrosis. *Matrix Biology Plus*, *3*, 100009. https://doi.org/10.1016/J.MBPLUS.2019.100009

Rastrick, J., & Birrell, M. (2014). The role of the inflammasome in fibrotic respiratory diseases. *Minerva Medica*, *105*(1), 9–23. https://doi.org/10.1002/PATH.4237

Riser, B. L., Barnes, J. L., & Varani, J. (2015). Balanced regulation of the CCN family of matricellular proteins: a novel approach to the prevention and treatment of fibrosis and cancer. *Journal of Cell Communication and Signaling*, *9*(4), 327. https://doi.org/10.1007/s12079-015-0309-3

Riser, B. L., Najmabadi, F., Garchow, K., Barnes, J. L., Peterson, D. R., & Sukowski, E. J. (2014). Treatment with the Matricellular Protein CCN3 Blocks and/or Reverses Fibrosis Development in Obesity with Diabetic Nephropathy. *American Journal of Pathology*, *184*(11), 2908–2921. https://doi.org/10.1016/j.ajpath.2014.07.009

Riser, B. L., Najmabadi, F., Perbal, B., Peterson, D. R., Rambow, J. A., Riser, M. L., Sukowski, E., Yeger, H., & Riser, S. C. (2009). CCN3 (NOV) is a negative regulator of CCN2 (CTGF) and a novel endogenous inhibitor of the fibrotic

pathway in an in vitro model of renal disease. *American Journal of Pathology*, *174*(5), 1725–1734. https://doi.org/10.2353/ajpath.2009.080241

Robinson, M.D., McCarthy, D.J., Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics, 26(1), 139-140. https://doi.org/10.1093/bioinformatics/btp616

Russo, B., Borowczyk, J., Boehncke, W.-H., Truchetet, M.-E., Modarressi, A., Brembilla, N. C., & Chizzolini, C. (2021). Dysfunctional Keratinocytes Increase Dermal Inflammation in Systemic Sclerosis: Results From Studies Using Tissue-Engineered Scleroderma Epidermis. *Arthritis & Rheumatology*, *73*(7), 1311–1317. https://doi.org/10.1002/ART.41659

Sahin, H., & Wasmuth, H. E. (2013). Chemokines in tissue fibrosis. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1832*(7), 1041–1048. https://doi.org/10.1016/J.BBADIS.2012.11.004

Schulz, J. N., Plomann, M., Sengle, G., Gullberg, D., Krieg, T., & Eckes, B. (2018). New developments on skin fibrosis - Essential signals emanating from the extracellular matrix for the control of myofibroblasts. *Matrix Biology*, *68–69*, 522–532. https://doi.org/10.1016/J.MATBIO.2018.01.025

Shi-wen, X., Racanelli, M., Ali, A., Simon, A., Quesnel, K., Stratton, R. J., & Leask, A. (2021). Verteporfin inhibits the persistent fibrotic phenotype of lesional scleroderma dermal fibroblasts. *Journal of Cell Communication and Signaling*, *15*(1), 71. https://doi.org/10.1007/S12079-020-00596-X

Solé-Boldo, L., Raddatz, G., Schütz, S., Mallm, J.-P., Rippe, K., Lonsdorf, A. S., Rodríguez-Paredes, M., & Lyko, F. (2020). Single-cell transcriptomes of the human skin reveal age-related loss of fibroblast priming. *Communications Biology*, *3*(1), 1–12. https://doi.org/10.1038/s42003-020-0922-4

Sonnylal, S., Shi-Wen, X., Leoni, P., Naff, K., van Pelt, C. S., Nakamura, H., Leask, A., Abraham, D., Bou-Gharios, G., & de Crombrugghe, B. (2010). Selective Expression of Connective Tissue Growth Factor in Fibroblasts In Vivo Promotes Systemic Tissue Fibrosis. *Arthritis & Rheumatology*, *62*(5), 1–17. https://doi.org/10.1002/art.27382

Taki, Z., Gostjeva, E., Thilly, W., Yaseen, B., Lopez, H., Mirza, M., Hassuji, Z., Vigneswaran, S., Abdi, B. A., Hart, A., Arumalla, N., Thomas, G., Denton, C. P., Suleman, Y., Liu, H., Venturini, C., O'Reilly, S., Xu, S., & Stratton, R. (2020). Pathogenic Activation of Mesenchymal Stem Cells Is Induced by the Disease Microenvironment in Systemic Sclerosis. *Arthritis & Rheumatology*, *72*(8), 1361–1374. https://doi.org/10.1002/ART.41267

Thannickal, V. J., Lee, D. Y., White, E. S., Cui, Z., Larios, J. M., Chacon, R., Horowitz, J. C., Day, R. M., & Thomas, P. E. (2003). Myofibroblast differentiation by transforming growth factor-β1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *Journal of Biological Chemistry*, *278*(14), 12384–12389. https://doi.org/10.1074/jbc.M208544200

Tsang, M., & Leask, A. (2014). CCN2 is required for recruitment of Sox2-expressing cells during cutaneous tissue repair. *Journal of Cell Communication and Signaling*, *9*(4), 341–346. https://doi.org/10.1007/S12079-014-0245-7

Tsang, M., Quesnel, K., Vincent, K., Hutchenreuther, J., Postovit, L.-M., & Leask, A. (2019). Insights into Fibroblast Plasticity: Cellular Communication Network 2 Is Required for Activation of Cancer-Associated Fibroblasts in a Murine Model of Melanoma. *American Journal of Pathology*, *190*(1), 206–221. https://doi.org/10.1016/J.AJPATH.2019.09.006

Valenzi, E., Bulik, M., Tabib, T., Morse, C., Sembrat, J., Bittar, H. T., Rojas, M., & Lafyatis, R. (2019). Single-cell analysis reveals fibroblast heterogeneity and myofibroblasts in systemic sclerosis-associated interstitial lung disease. *Annals of the Rheumatic Diseases*, *78*(10), 1387. https://doi.org/10.1136/ANNRHEUMDIS-2018-214865

Vorstandlechner, V., Laggner, M., Kalinina, P., Haslik, W., Radtke, C., Shaw, L., Lichtenberger, B. M., Tschachler, E., Ankersmit, H. J., & Mildner, M. (2020). Deciphering the functional heterogeneity of skin fibroblasts using single-cell RNA sequencing. *FASEB Journal*, *34*(3), 3677–3692. https://doi.org/10.1096/FJ.201902001RR

Walraven, M., & Hinz, B. (2018). Therapeutic approaches to control tissue repair and fibrosis: Extracellular matrix as a game changer. *Matrix Biology*, *71–72*, 205–224. https://doi.org/10.1016/J.MATBIO.2018.02.020

Wernig, G., Chen, S.-Y., Cui, L., van Neste, C., Tsai, J. M., Kambham, N., Vogel, H., Natkunam, Y., Gilliland, D. G., Nolan, G., & Weissman, I. L. (2017). Unifying mechanism for different fibrotic diseases. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(18), 4757–4762. https://doi.org/10.1073/PNAS.1621375114

Wu, M., Melichian, D. S., de La Garza, M., Gruner, K., Bhattacharyya, S., Barr, L., Nair, A., Shahrara, S., Sporn, P. H. S., Mustoe, T. A., Tourtellotte, W. G., & Varga, J. (2009). Essential Roles for Early Growth Response Transcription Factor Egr-1 in Tissue Fibrosis and Wound Healing. *American Journal of Pathology*, *175*, 1041–1055. https://doi.org/10.2353/ajpath.2009.090241

Yamamoto, T. (2017). Animal Models of Systemic Sclerosis. In *Animal Models for the Study of Human Disease*. https://doi.org/10.1016/B978-0-12-809468-6.00036-X

Yamamoto, T. (2006). The bleomycin-induced scleroderma model: what have we learned for scleroderma pathogenesis? *Archives of Dermatological Research*, *297*(8), 333–344. https://doi.org/10.1007/S00403-005-0635-Z

Yamamoto, T., & Katayama, I. (2011). Vascular Changes in Bleomycin-Induced Scleroderma. *International Journal of Rheumatology*, *2011*, 1–5. https://doi.org/10.1155/2011/270938

Yamamoto, T., Takagawa, S., Katayama, I., Yamazaki, K., Hamazaki, Y., Shinkai, H., & Nishioka, K. (1999). Animal model of sclerotic skin. I: Local injections of

bleomycin induce sclerotic skin mimicking scleroderma. *Journal of Investigative Dermatology*. https://doi.org/10.1046/j.1523-1747.1999.00528.x

Chapter 5

5 Discussion

5.1 Overview

The overall objective of my thesis was to investigate the expression and contribution of CCN proteins in dermal fibrosis and to determine if therapeutic peptides based on CCN3 represent a novel anti-fibrotic strategy. Since their discovery in the early 1990s, CCN proteins have been shown to play a role in a wide range of cellular processes including: cell adhesion, migration, proliferation, differentiation, survival, and apoptosis (Lau, 2016; Perbal, 2004). Thus, it is not surprising that they have been implicated in several pathological conditions, including: inflammation, retinopathy, cancer, and fibrotic conditions such SSc (Leask, 2020b; Riser et al., 2015). SSc is a chronic disease characterized by vascular abnormalities, dysregulated inflammation, and diffuse fibrosis of the skin and internal organs (Varga & Abraham, 2007). Although much progress has been made in deciphering the complex pathophysiology of SSc, there is still no viable disease-modifying treatment, and patients often experience significant morbidity and mortality (Cutolo et al., 2019). In SSc, CCN1 and CCN2 are upregulated in fibrotic lesions of early-onset patients with diffuse cutaneous disease (Holmes et al., 2003; Quesnel et al., 2019). CCN1 likely plays a role in mediating the formation of organized collagen fibres and may contribute to early inflammatory changes (Quesnel et al., 2019). Fibroblast-specific expression of CCN2 plays a role in promoting a pro-fibrotic microenvironment through the recruitment and sustained activation of myofibroblasts (Liu et al., 2011; Tsang et al., 2019). On the other hand, CCN3 is reciprocally regulated to CCN1 and CCN2 in multiple cell types, and has anti-fibrotic effects opposing CCN2 in renal fibrosis (Riser et al., 2014). This prompted BLR Bio, a start-up company, to develop a proprietary CCN3-based peptide, BLR-200, that mirrors the anti-fibrotic effects of full length CCN3 (U.S. Patent No. 9114112B2, 2015). Thus, targeting the activity and regulation of CCN1 and CCN2 indirectly, for example, by using BLR-200, may represent a novel therapeutic approach for SSc.

Despite substantial available evidence of CCN protein involvement in fibrosis, there is still a considerable gap in knowledge. The exact mechanisms by which CCN proteins are regulated, and function *in vivo* remains elusive. Many studies on CCN protein activity have been conducted *in vitro* using 2-dimensional cell culture models. Although these studies can be useful to decipher simple regulatory mechanisms, they often lack significance in a biological context, as CCN protein activity is crucially dependent on the 3-dimensional composition of the cellular microenvironment (Leask, 2020b). It is imperative to use animal models to truly understand the physiological function of this unique family of matricellular proteins. Furthermore, few studies have focused on the specific anti-fibrotic role of CCN3 in dermal fibrosis. The aim of my thesis is to contribute to these gaps in knowledge.

5.2 Regulation of CCN Proteins in Dermal Fibroblasts

The first study reported in my thesis investigates the reciprocal regulation of CCN1, CCN2, and CCN3 in human dermal fibroblasts in an *in vitro* model of fibrosis. In this series of experiments, I used TGFβ1 to stimulate human dermal fibroblasts, and simultaneously monitored the mRNA and protein expression of CCN1, CCN2, and CCN3. I also used chemical signal transduction inhibitors to identify common pathways mediating the effects of TGFβ1 on CCN1, CCN2, and CCN3 expression. My findings confirmed that mRNA expression of *CCN1* and *CCN2* are reciprocally regulated to *CCN3* in response to TGFβ1 in human dermal fibroblasts. Furthermore, my data suggest that *CCN1/CCN2* mRNA expression and CCN1/CCN2 protein levels are induced via a similar pro-adhesive pathway involving FAK, MEK1, TAK1, and YAP1. Conversely, *CCN3* mRNA is reduced by TGFβ1 through a divergent pathway, that does not involve MEK1, YAP1, or TAK1. These data are consistent with literature showing that CCN1 and CCN2 are expressed in a yin/yang fashion opposite to CCN3 (Abd El Kader et al., 2012; Riser et al., 2010, 2015). These results contribute to the overall understanding of CCN protein regulation and provide implications for CCN protein regulation in dermal fibrosis *in vivo*. These results are also consistent with the notion that non-canonical pro-adhesive TGFβ signaling promotes a fibrogenic response in fibroblasts (Chaqour, 2020; Leask, 2020a). Moreover, the ability of a YAP1 inhibitor to prevent TGFβ1-induced CCN1 and CCN2 expression

also sheds new light on the involvement of YAP1 in mediating the pro-fibrotic effects of TGFβ in dermal fibroblasts.

An unexpected problem I encountered during this study was the inability to detect CCN3 protein by Western blot, either in the presence or absence of TGFβ1. However, I was able to detect CCN3 protein in NCI-H295R cells, a tumor cell line that expresses CCN3 (Gazdar et al., 1990; Kyurkchiev et al., 2004), indicating the validity of my methods. Based on this, and previous literature, I concluded that CCN3 protein is not basally expressed in proliferating human dermal fibroblasts. Previous studies have shown that low levels of intracellular CCN3 are produced in fibroblasts in cultures, however, in growing cells the expression of CCN3 protein is quickly downregulated (Scholz et al., 1996). To examine the potential role of CCN3 in regulating fibrogenic responses, it was necessary, therefore, for me to generate CCN3-overexpressing human dermal fibroblasts using lentiviral transduction.

In CCN3-overexpressing human dermal fibroblasts, the ability of TGFβ1 to induce CCN2 protein expression was significantly impaired. Consistent with previous reports showing that CCN2-deficient dermal fibroblasts retained TGFβ-responsiveness *in vitro* (Liu et al., 2011), CCN3 overexpression had no significant effect on the ability of TGFβ1 to induce mRNA expression of the pro-fibrotic biomarkers endothelin-1 and integrin alpha 11. Since *in vivo* loss or inhibition of CCN2 expression severely impairs fibrogenesis, including myofibroblast activation, in a TGFβ-independent manner (Chen et al., 2006; Holmes et al., 2001), studying the full activity of CCN proteins in animal models is imperative.

In summation, the data from this report provide valuable insights into the coordinated and opposite regulation of CCN1, CCN2, and CCN3 in human dermal fibroblasts. Furthermore, I have shown that CCN3 may have inhibitory effects on CCN2, thus supporting *in vivo* testing of the anti-fibrotic effects of CCN3, for example, with CCN3-based peptides.

5.3 Evaluating CCN3-Based Peptides in an Animal Model of SSc Dermal Fibrosis

One of the most representative murine models of SSc is the bleomycin-induced model. In this model, daily subcutaneous bleomycin injection induces an early inflammatory response characterized by the presence of mononuclear and mast cells, resembling early inflammatory changes in SSc (Yamamoto et al., 1999). Early immune infiltration in the lesional dermis results in an inflammatory microenvironment that drives fibrosis. This inflammatory response, however, is short-lived and diminishes after 1-2 weeks of daily injection, at which point, progressive fibrosis occurs in the dermis. After 3-4 weeks of daily injection, dermal thickness increases, with excessive accumulation of collagen and appearance of αSMA-expressing myofibroblasts, mimicking the fibrotic features of lesional SSc fibrosis (Yamamoto, 2017). Thus, the bleomycin-induced model provides a valuable tool to investigate fibrotic changes at multiple stages that resemble those seen in patients with SSc.

In my thesis, I chose to investigate the effect of the CCN3-based peptide, BLR-200, at two separate timepoints. First, I investigated the ability of BLR-200 to prevent progressive dermal fibrosis 28 days post-initiation of bleomycin injection. This allows for investigation of the overall anti-fibrotic ability of BLR-200. Second, I investigated the ability of BLR-200 to prevent inflammatory changes 10 days post-initiation of bleomycin injection. At this timepoint, the effect of BLR-200 on pro-inflammatory and pro-fibrotic responses to bleomycin-induced fibrosis can be assessed. Both timepoints can provide valuable insight into the potential anti-fibrotic activity of CCN3-based peptides in SSc dermal fibrosis.

5.3.1 CCN3-Based Peptides Prevent Bleomycin-Induced Fibrosis

In this experiment, bleomycin or PBS was injected subcutaneously into the flank of mice once daily for 28 days. Bleomycin-treated mice were further divided into two treatment groups, which received intraperitoneal injections of either BLR-200 or a scrambled peptide. After 28 days, dermal tissue was collected and analyzed by histology, qPCR, and proteomics. In my initial analysis, I found that BLR-200 treatment prevented the bleomycin-induced increase in skin thickness and collagen deposition. Furthermore, qPCR

analysis revealed that BLR-200 prevented expression of mRNAs encoding the collagen the cross-linking genes *Plod2* and *Lox*. These genes are involved in promoting stably cross-linked collagen strands and are upregulated in multiple fibrotic disorders (Chen et al., 2018; van der Slot et al., 2003). I next analyzed expression of αSMA, the prototypical marker of activated myofibroblasts (Desmoulière et al., 1993). Based on histological and qPCR analysis, I found that BLR-200 treatment prevented the bleomycin-induced increase in αSMA-expressing myofibroblasts in the dermis.

I next wanted to investigate CCN1 and CCN2 expression via histology and qPCR. My data indicate that BLR-200 effectively prevented the bleomycin-induced increase in *Ccn1* and *Ccn2* mRNA expression. Similarly, the number of CCN1- and CCN2-positive cells in the dermis were prevented. This is consistent with my previous finding that CCN3 overexpression inhibited CCN2 protein expression in dermal fibroblasts. Moreover, these data indicate that, *in vivo*, CCN3 may also have inhibitory effects on CCN1 expression. My results are also consistent with literature showing that loss of CCN2 impairs skin thickening, collagen deposition, and appearance of αSMA-expressing cells in bleomycin-induced fibrosis (Liu et al., 2011, 2014). This is the first study to report, in an *in vivo* model of dermal fibrosis, that CCN3-based treatment can regulate CCN1 and CCN2 expression. It is not known whether this effect is direct or indirect, thus mechanistic studies should be employed in the future. For example, future studies investigating the potential binding partners of BLR-200 would be extremely useful in deciphering this issue. Protein binding assays, including library screening of potential binding partners for BLR-200 would provide valuable information. These experiments would allow us to determine if BLR-200 acts by direct binding to CCN1 and/or CCN2, or by binding to other factors that prevent CCN1 and/or CCN2 expression and activity. Of course, it is also possible that BLR-200 acts through a completely different anti-fibrotic mechanism.

Collectively, my data reveal that BLR-200 potently impairs bleomycin-induced skin fibrosis, as demonstrated by prevention of dermal thickening, collagen deposition, activated αSMA-expressing myofibroblasts, and expression of CCN1 and CCN2.

To expand my investigation, I collaborated with colleagues from the Proteomic Resource Facility (PRF) in the Department of Pathology at the University of Michigan to employ proteomic analysis of skin samples using mass spectrometry-based Tandem Mass Tag. Proteomic analysis of whole skin samples revealed that BLR-200 prevented several proteins involved in ECM organization (fibromodulin, nectin-2, pecam-1) and pro-fibrotic laminin interactions (lama5, lama3, lamb3, lamc2). The use of this analytical approach offers valuable unbiased analysis of the dermal proteome and provides further evidence of the anti-fibrotic effect of BLR-200.

I also analyzed expression of the progenitor cell marker SOX2. Previous studies have shown that collagen-lineage dermal fibroblasts, through a SOX2-expressing progenitor cell-like intermediate, contribute to the activated αSMA-expressing myofibroblast population in bleomycin-induced fibrosis (Liu et al., 2014; Tsang et al., 2019). Furthermore, CCN2 is required for recruitment and activation of this SOX2-positive myofibroblast population (Tsang & Leask, 2014). My results indicate that BLR-200 prevented the bleomycin-induced increase in *Sox2* mRNA expression and reduced the number of SOX2-positive cells in the fibrotic reticular dermis. This finding is consistent with my observation that BLR-200 can prevent activated αSMA-expressing myofibroblasts in the fibrotic lesion. Thus, my data indicate that BLR-200 treatment likely impairs the CCN2-dependent recruitment and activation of collagen-lineage dermal fibroblasts in bleomycin-induced dermal fibrosis.

I also show that BLR-200 did not have any significant effects on the expression of the mechanosensitive transcriptional cofactor YAP1. This was unexpected, as YAP1 has been shown to respond to and mediate the pro-adhesive signaling pathway in active myofibroblasts (Shi-wen et al., 2021). Since my data also show that expression of YAP1 target genes including *Ccn1*, *Ccn2*, and *Acta2* (αSMA) were prevented by BLR-200, it appears that BLR-200's anti-fibrotic effects are either downstream or independent of YAP1 signaling.

The overall results of the second report in my thesis demonstrate that therapeutic treatment with a CCN3-based peptide prevents fibrotic changes in the bleomycin-induced model of

SSc dermal fibrosis. At this time, this is the first study demonstrating that targeting CCN proteins using a CCN3-based therapeutic approach prevents fibrotic changes in an animal model of dermal fibrosis. These results represent an exciting discovery in the field and warrant further studies to determine the exact anti-fibrotic mechanisms of CCN3 in SSc dermal fibrosis.

For example, an alternative pre-clinical method to investigate BLR-200's ability to prevent SSc fibrosis would be to test the peptides in a 3-dimensional cell culture model of human SSc. Advancements in 3-dimensional cell culture technologies have made it possible to develop advanced physiologically relevant 3-dimensional models of SSc *in vitro* (De Pieri et al., 2021). A 3D model examining the response of patient-derived SSc fibroblasts to BLR-200 treatment would provide ample valuable information. This experiment would allow us to examine the direct effect of BLR-200 on SSc fibroblasts. For instance, one could determine if BLR-200 treatment results trans-differentiation, deactivation, or potentially apoptosis of these fibroblasts. Furthermore, one could test if the effects of BLR-200 on SSc fibroblasts are transient or permanent. All of these insights would have significant clinical impact.

5.3.2 CCN3-Based Peptides Impair Fibroblast Response to The Inflammatory Microenvironment

In the final study reported in my thesis, I used unbiased proteomic and scRNA-seq analysis to investigate the ability of BLR-200 to suppress specific early inflammatory and pro-fibrotic responses in bleomycin-induced fibrosis after 10 days. The animal experiments were performed exactly the same as my previous report, except tissue samples were collected 10 days post-initiation of bleomycin injection.

Once again, I collaborated with colleagues from the PRF at the University of Michigan to analyze overall proteomic changes using mass spectrometry. I found that BLR-200 prevented the bleomycin-induced increase in markers of keratinocyte activation including keratin1, keratin6, keratin14, and keratin15. Keratinocyte activation represents an early inflammatory response in the epidermis of fibrotic lesions, including in SSc (Aden et al., 2008; Nikitorowicz-Buniak et al., 2014). I also found that BLR-200 prevented markers of

myofibroblast activation including fibrillin-1, dermatopontin, tropomyosin, and myosin light chain 6b (Fujimura et al., 2011; Kissin et al., 2002; Malmstrom et al., 2004; Okamoto & Fujiwara, 2009). Thus, my results indicate that BLR-200 inhibits inflammation-driven pro-fibrotic protein expression observed 10 days post-initiation of bleomycin injection. Considering that, in SSc, keratinocyte activation precedes fibrotic changes in the microenvironment and promote myofibroblast activation (Aden et al., 2010; McCoy et al., 2017), it is likely that fibroblasts in the fibrotic lesion are in the early stages of activation after 10 days of bleomycin injection. Given this observation, I next wanted to investigate the transcriptomic changes occurring in the activating fibroblasts at this point, and the effect of BLR-200 on these changes.

Thus, to further investigate the ability of BLR-200 to prevent early inflammatory and pro-fibrotic responses in bleomycin-induced fibrosis, I used scRNA-seq to investigate transcriptomic changes in the activating collagen-lineage fibroblast population. The use of scRNA-seq provides an unbiased approach to decipher fibroblast heterogeneity and identify gene signatures capable of predicting biological activity (Haque et al., 2017). In this report, I used a transgenic mouse line to postnatally label *Col1a2*-expressing resident dermal fibroblasts with membrane-targeted GFP (Bou-Gharios et al., 1996; Denton et al., 2001). This population is known to become activated and contribute to the myofibroblast population in bleomycin-induced fibrosis (Tsang et al., 2019). Thus, labelling these "pre-myofibroblasts" allows for investigation of cellular changes in cells undergoing myofibroblast activation in response to the inflammatory microenvironment. At the conclusion of the experiment, fibroblasts were isolated from the dorsal skin of the mice, and collagen-lineage (GFP+) cells were sorted by FACS. These cells were then subjected to scRNA-seq. As expected, my initial scRNA-seq analysis revealed that collagen-lineage myofibroblasts exhibited considerable heterogeneity in bleomycin-induced fibrosis. Among the treatment groups, I identified 7 different fibroblast subpopulations that could be roughly classified into 3 major groups, including an inflammatory subset, a secretory-reticular subset, and a mesenchymal-like subset.

Next, I investigated the effects of BLR-200 on these fibroblast subpopulations. I found that BLR-200 impaired a bleomycin-induced transcriptomic shift in the inflammatory

fibroblast subpopulation (IF1). Upon investigation of the genes responsible for this shift, I found that BLR-200 impaired the bleomycin-induced increase in *Il6* and *Cxcl2*, both of which have been implicated in early fibrogenesis in SSc (Feghali et al., 1992; Johnson et al., 2015). I also found that BLR-200 treatment prevented the bleomycin-induced overexpression of NLRP3 inflammasome markers, including *Il18, Tlr2, Il1r1*, and *Il1r2*. Inflammasome activation is an early inflammatory event in fibrogenesis in multiple organs, including the lung, kidney, liver, and dermis (Colak et al., 2021; Hsu et al., 2021; Rastrick & Birrell, 2014). Furthermore, an inflammasome-related gene signature is overexpressed in SSc and may play a role in myofibroblast activation and excessive collagen deposition (Artlett et al., 2011; Martinez-Godinez et al., 2015). Thus, my data indicate that BLR-200 can impair early pro-inflammatory changes implicated in creating a pro-fibrotic microenvironment.

Another interesting finding in this report was that BLR-200 prevented the bleomycin-induced increase in *Egr1* expression in the secretory-reticular (SC2) fibroblast subpopulation. EGR1, a transcription factor that mediates early cellular responses to injury, is also overexpressed in the fibrotic tissue of SSc patients (Bhattacharyya, Wu, et al., 2011). Moreover, EGR1 overexpression is implicated in an inflammatory subset of SSc patients, indicating its potential role in mediating early inflammatory events in fibrogenesis (Bhattacharyya, Sargent, et al., 2011). Interestingly, BLR-200 treatment failed to prevent the bleomycin-induced increase in expression of other pro-fibrotic transcription factors, including *Fosb* and *Junb* (Avouac et al., 2012). This is consistent with the notion that BLR-200 has very specific anti-fibrotic effects.

Finally, my scRNA-seq analysis revealed that BLR-200 prevented the appearance of a mesenchymal fibroblast subpopulation. This mesenchymal subpopulation was characterized by enrichment of functional pathways involved in development and differentiation (Solé-Boldo et al., 2020). Previous studies have reported that a mesenchymal-like fibroblast subpopulation contributes to dermal fibrosis, and this population may actually be primed to over-respond to fibrotic stimuli (Deng et al., 2021; Taki et al., 2020). Therefore, BLR-200 may be preventing the appearance of a highly pro-

fibrotic subpopulation. However, the exact role of this mesenchymal cluster in my study is not known and requires further investigation.

To date, scRNA-seq studies have mainly focussed on dissecting fibroblast heterogeneity in dermal fibrosis (Ascensión et al., 2021; Deng et al., 2021; Philippeos et al., 2018; Solé-Boldo et al., 2020). While this provides useful information, very few studies have investigated this heterogeneity in response to a pro-inflammatory, pro-fibrotic microenvironment. Thus, my thesis greatly contributes to this gap in knowledge. My results also emphasize the important contribution of collagen-expressing dermal fibroblasts to the heterogenous fibroblast subpopulation in bleomycin-induced dermal fibrosis. Furthermore, I have shown that therapeutic peptides based on CCN3 impair early pro-inflammatory and pro-fibrotic responses in this collagen-lineage fibroblast population.

It is important to note that the bleomycin-induced model of SSc, while one of the more useful tools for studying SSc fibrosis, is still an animal model. Thus, it is a simplification of a much more complex human disorder. Nevertheless, the findings within my thesis still provide novel information on the pathogenic mechanisms of fibrosis and will potentially contribute to the overall understanding of mechanisms underlying human SSc.

The data generated during my thesis represent a valuable starting point for an extensive study aimed at full assessment of the ability of BLR-200 to prevent fibrosis. Since fibrogenesis is a highly spatially and temporally regulated process, investigating multiple different timepoints in the bleomycin-induced model would provide greater insight into the exact anti-fibrotic effects of BLR-200. For example, investigating changes 5 days post-initiation of bleomycin injection would allow us to determine the effect of BLR-200 on the robust inflammatory response that occurs immediately following vascular injury. Furthermore, investigating changes 15 days post-initiation of bleomycin injection would provide insight on BLR-200's effects during the transition from the inflammatory phase to the actively fibrotic phase of the model. A longitudinal study of this nature would likely reveal the contribution and fate of the observed mesenchymal-like fibroblast subpopulation. Moreover, while my scRNA-seq studies provide valuable, novel information on fibroblast heterogeneity in bleomycin-induced dermal fibrosis,

investigation of multiple timepoints would greatly build upon characterization of this model. This would have a significant impact on our overall understanding of fibrosis and positively impact pre-clinical testing of other potential anti-fibrotic therapies.

Investigating the effect of BLR-200 on established fibrosis will also uncover valuable therapeutic information. My thesis focusses on the ability of BLR-200 to prevent fibrosis and impair the early response to inflammation. This is clinically important, especially in early dcSSc, when fibrogenesis is actively occurring (Asano, 2018). However, future studies should aim at determining if BLR-200 can reverse established fibrosis in the bleomycin-induced model of SSc. In this experiment, BLR-200 injections would begin at 28 days post-initiation of bleomycin injection, once dermal fibrosis has been established. If BLR-200 can promote resolution or reversal of fibrosis, this would have a substantial impact on treatment of SSc and fibrotic diseases in general.

5.4 Summary

In summation, I have shown that, in dermal fibroblasts, CCN1 and CCN2 are reciprocally regulated to CCN3 through differential use of non-canonical TGFβ signaling pathways. Furthermore, I have shown that CCN3-based peptides treat fibrosis in an animal model of SSc dermal fibrosis and impair the ability of collagen-expressing fibroblasts to respond to the inflammatory microenvironment. Thus, my thesis provides important evidence supporting the therapeutic potential of CCN3-based peptides in treatment of fibrotic disorders, a finding that may have significant clinical impact.

5.5 References

Abd El Kader, T., Kubota, S., Janune, D., Nishida, T., Hattori, T., Aoyama, E., Perbal, B., Kuboki, T., & Takigawa, M. (2012). Anti-fibrotic effect of CCN3 accompanied by altered gene expression profile of the CCN family. *Journal of Cell Communication and Signaling*, *7*(1), 11–18. https://doi.org/10.1007/S12079-012-0180-4

Aden, N., Nuttall, A., Shiwen, X., Winter, P. de, Leask, A., Black, C. M., Denton, C. P., Abraham, D. J., & Stratton, R. J. (2010). Epithelial Cells Promote Fibroblast Activation via IL-1α in Systemic Sclerosis. *Journal of Investigative Dermatology*, *130*(9), 2191–2200. https://doi.org/10.1038/JID.2010.120

Aden, N., Shiwen, X., Aden, D., Black, C., Nuttall, A., Denton, C. P., Leask, A., Abraham, D., & Stratton, R. (2008). Proteomic analysis of scleroderma lesional skin reveals

activated wound healing phenotype of epidermal cell layer. *Rheumatology*, *47*(12), 1754–1760. https://doi.org/10.1093/RHEUMATOLOGY/KEN370

Artlett, C. M., Sassi-Gaha, S., Rieger, J. L., Boesteanu, A. C., Feghali-Bostwick, C. A., & Katsikis, P. D. (2011). The inflammasome activating caspase 1 mediates fibrosis and myofibroblast differentiation in systemic sclerosis. *Arthritis and Rheumatism*, *63*(11), 3563–3574. https://doi.org/10.1002/ART.30568

Asano, Y. (2018). Systemic sclerosis. In *Journal of Dermatology*. https://doi.org/10.1111/1346-8138.14153

Ascensión, A. M., Fuertes-Álvarez, S., Ibañez-Solé, O., Izeta, A., & Araúzo-Bravo, M. J. (2021). Human Dermal Fibroblast Subpopulations Are Conserved across Single-Cell RNA Sequencing Studies. *Journal of Investigative Dermatology*, *141*(7), 1744. https://doi.org/10.1016/J.JID.2020.11.028

Avouac, J., Palumbo, K., Tomcik, M., Zerr, P., Dees, C., Horn, A., Maurer, B., Akhmetshina, A., Beyer, C., Sadowski, A., Schneider, H., Shiozawa, S., Distler, O., Schett, G., Allanore, Y., & Distler, J. H. W. (2012). Inhibition of activator protein 1 signaling abrogates transforming growth factor β–mediated activation of fibroblasts and prevents experimental fibrosis. *Arthritis & Rheumatology*, *64*(5), 1642–1652. https://doi.org/10.1002/ART.33501

Bhattacharyya, S., Sargent, J. L., Lin, S., Tourtellotte, W. G., Takehara, K., Whitfield, M. L., & Varga, J. (2011). Egr-1 Induces a Profibrotic Injury/Repair Gene Program Associated with Systemic Sclerosis. *PLoS ONE*, *6*(9), e23082. https://doi.org/10.1371/journal.pone.0023082

Bhattacharyya, S., Wu, M., Fang, F., Tourtellotte, W., Feghali-Bostwick, C., & Varga, J. (2011). Early growth response transcription factors: key mediators of fibrosis and novel targets for anti-fibrotic therapy. *Matrix Biology*, *30*(4), 242. https://doi.org/10.1016/J.MATBIO.2011.03.005

Bou-Gharios, G., Garrett, L. A., Rossert, J., Niederreither, K., Eberspaecher, H., Smith, C., Black, C., & Crombrugghe, B. (1996). A potent far-upstream enhancer in the mouse pro alpha 2(I) collagen gene regulates expression of reporter genes in transgenic mice. *Journal of Cell Biology*, *134*(5), 1333–1344. https://doi.org/10.1083/JCB.134.5.1333

Chaqour, B. (2020). Caught between a “Rho” and a hard place: are CCN1/CYR61 and CCN2/CTGF the arbiters of microvascular stiffness? *Journal of Cell Communication and Signaling*, *14*(1), 29. https://doi.org/10.1007/S12079-019-00529-3

Chen, L., Li, S., & Li, W. (2018). LOX/LOXL in pulmonary fibrosis: potential therapeutic targets. *Journal of Drug Targeting*, *27*(7), 790–796. https://doi.org/10.1080/1061186X.2018.1550649

Chen, Y., Shi-Wen, X., Eastwood, M., Black, C. M., Denton, C. P., Leask, A., Abraham, D. J., Xu, S. W., Eastwood, M., Black, C. M., Denton, C. P., Leask, A., & Abraham, D. J. (2006). Contribution of activin receptor-like kinase 5 (transforming growth factor β receptor type I) signaling to the fibrotic phenotype of scleroderma

fibroblasts. *Arthritis & Rheumatology*, *54*(4), 1309–1316. https://doi.org/10.1002/art.21725

Colak, Y., Hasan, B., Erkalma, B., Tandon, K., Zervos, X., Menzo, E. lo, & Erim, T. (2021). Pathogenetic mechanisms of nonalcoholic fatty liver disease and inhibition of the inflammasome as a new therapeutic target. *Clinics and Research in Hepatology and Gastroenterology*, *45*(4), 101710. https://doi.org/10.1016/J.CLINRE.2021.101710

Cutolo, M., Soldano, S., & Smith, V. (2019). Pathophysiology of systemic sclerosis: current understanding and new insights. *Expert Review of Clinical Immunology*, *15*(7), 753–764. https://doi.org/10.1080/1744666X.2019.1614915

Deng, C.-C., Hu, Y.-F., Zhu, D.-H., Cheng, Q., Gu, J.-J., Feng, Q.-L., Zhang, L.-X., Xu, Y.-P., Wang, D., Rong, Z., & Yang, B. (2021). Single-cell RNA-seq reveals fibroblast heterogeneity and increased mesenchymal fibroblasts in human fibrotic skin diseases. *Nature Communications*, *12*(3709), 1–16. https://doi.org/10.1038/s41467-021-24110-y

Denton, C. P., Zheng, B., Shiwen, X., Zhang, Z., Bou-Gharios, G., Eberspaecher, H., Black, C. M., & de Crombrugghe, B. (2001). Activation of a Fibroblast-Specific Enhancer of the Pro2(I) Collagen Gene in Tight-Skin Mice. *Arthritis & Rheumatology*, *44*(3), 712–722. https://doi.org/10.1002/1529-0131

Desmoulière, A., Geinoz, A., Gabbiani, F., & Gabbiani, G. (1993). Transforming growth factor-β 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *Journal of Cell Biology*, *122*(1), 103–111. https://doi.org/10.1083/JCB.122.1.103

Driskell, R. R., & Watt, F. M. (2015). Understanding fibroblast heterogeneity in the skin. *Trends in Cell Biology*, *25*(2), 92–99. https://doi.org/10.1016/J.TCB.2014.10.001

Feghali, C., Bost, K., Boulware, D., & Levy, L. (1992). Mechanisms of pathogenesis in scleroderma. I. Overproduction of interleukin 6 by fibroblasts cultured from affected skin sites of patients with scleroderma. *The Journal of Rheumatology*, *19*(8), 1207–1211. https://pubmed.ncbi.nlm.nih.gov/1404155/

Fujimura, T., Hotta, M., Kitahara, T., & Takema, Y. (2011). Loss of contraction force in dermal fibroblasts with aging due to decreases in myosin light chain phosphorylation enzymes. *Archives of Pharmacal Research*, *34*(6), 1015–1022. https://doi.org/10.1007/S12272-011-0619-9

Gao, Y., Yao, X., Zhai, Y., Li, L., Li, H., Sun, X., Yu, P., Xue, T., Li, Y., & Hu, Y. (2021). Single cell transcriptional zonation of human psoriasis skin identifies an alternative immunoregulatory axis conducted by skin resident cells. *Cell Death & Disease*, *12*(5), 450. https://doi.org/10.1038/S41419-021-03724-6

Gazdar, A. F., Oie, H. K., Shackleton, C. H., Chen, T. R., Triche, T. J., Myers, C. E., Chrousos, G. P., Brennan, M. F., Stein, C. A., & la Rocca, R. v. (1990). Establishment and Characterization of a Human Adrenocortical Carcinoma Cell Line That Expresses Multiple Pathways of Steroid Biosynthesis. *Cancer Research*, *50*(17), 5488–5496.

Haque, A., Engel, J., Teichmann, S. A., & Lönnberg, T. (2017). A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications. *Genome Medicine*, *9*(1), 1–12. https://doi.org/10.1186/S13073-017-0467-4

Holmes, A., Abraham, D. J., Chen, Y., Denton, C., Shi-Wen, X., Black, C. M., & Leask, A. (2003). Constitutive Connective Tissue Growth Factor Expression in Scleroderma Fibroblasts Is Dependent on Sp1. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M305019200

Holmes, A., Abraham, D. J., Sa, S., Shiwen, X., Black, C. M., & Leask, A. (2001). CTGF and SMADs, Maintenance of Scleroderma Phenotype Is Independent of SMAD Signaling. *Journal of Biological Chemistry*, *276*(14), 10594–10601. https://doi.org/10.1074/jbc.M010149200

Hsu, Y.-H., Zheng, C.-M., Chou, C.-L., Chen, Y.-J., Lee, Y.-H., Lin, Y.-F., Chiu, H.-W., & Therapeutic, H. (2021). Therapeutic Effect of Endothelin-Converting Enzyme Inhibitor on Chronic Kidney Disease through the Inhibition of Endoplasmic Reticulum Stress and the NLRP3 Inflammasome. *Biomedicines*, *9*(398), 1–14. https://doi.org/10.3390/biomedicines9040398

Johnson, M. E., Mahoney, J. M., Taroni, J., Sargent, J. L., Marmarelis, E., Wu, M.-R., Varga, J., Hinchcliff, M. E., & Whitfield, M. L. (2015). Experimentally-Derived Fibroblast Gene Signatures Identify Molecular Pathways Associated with Distinct Subsets of Systemic Sclerosis Patients in Three Independent Cohorts. *PLoS ONE*, *10*(1), e0114017. https://doi.org/10.1371/JOURNAL.PONE.0114017

Kissin, E. Y., Lemaire, R., Korn, J. H., & Lafyatis, R. (2002). Transforming growth factor β induces fibroblast fibrillin-1 matrix formation. *Arthritis & Rheumatism*, *46*(11), 3000–3009. https://doi.org/10.1002/ART.10621

Kyurkchiev, S., Yeger, H., Bleau, A.-M., & Perbal, B. (2004). Potential cellular conformations of the CCN3(NOV) protein. *Cell Communication & Signaling*, *2*, 9. https://doi.org/10.1186/1478-811X-2-9

Lau, L. F. (2016). Cell surface receptors for CCN proteins. *Journal of Cell Communication and Signaling*, *10*(2), 127. https://doi.org/10.1007/S12079-016-0324-Z

Leask, A. (2020a). The hard problem: Mechanotransduction perpetuates the myofibroblast phenotype in scleroderma fibrosis. *Wound Repair and Regeneration*, *29*, 582–587. https://onlinelibrary-wiley-com.proxy1.lib.uwo.ca/doi/pdfdirect/10.1111/wrr.12889

Leask, A. (2020b). Conjunction junction, what's the function? CCN proteins as targets in fibrosis and cancers. *American Journal of Physiology - Cell Physiology*, *318*(6), C1046. https://doi.org/10.1152/AJPCELL.00028.2020

Liu, S., Herault, Y., Pavlovic, G., & Leask, A. (2014). Skin progenitor cells contribute to bleomycin-induced skin fibrosis. *Arthritis & Rheumatology*. https://doi.org/10.1002/art.38276

Liu, S., Shi-Wen, X., Abraham, D. J., & Leask, A. (2011). CCN2 is required for bleomycin-induced skin fibrosis in mice. *Arthritis & Rheumatology*, *63*(1), 239–246. https://doi.org/10.1002/art.30074

Liu, S., Shi-wen, X., Blumbach, K., Eastwood, M., Denton, C. P., Eckes, B., Krieg, T., Abraham, D. J., & Leask, A. (2010). Expression of integrin β1 by fibroblasts is required for tissue repair in vivo. *Journal of Cell Science*, *123*(21), 3674–3682. https://doi.org/10.1242/JCS.070672

Malmstrom, J., Linberg, H., Lindberg, C., Bratt, C., Wieslander, E., Delander, E. L., Särnstrand, B., Burns, J. S., Mose-Larsen, P., Fey, S., & Marko-Varga, G. (2004). Transforming Growth Factor-β1 Specifically Induce Proteins Involved in the Myofibroblast Contractile Apparatus. *Molecular & Cellular Proteomics*, *3*(5), 466–477. https://doi.org/10.1074/MCP.M300108-MCP200

Martinez-Godinez, M. A., Cruz-Dominguez, M. P., Jara, L. J., Dominguez-Lopez, A., Jarillo-Luna, R. A., Vera-Lastra, O., Montes-Cortes, D. H., Campos-Rodriguez, R., Lopez-Sanchez, D. M., Mejia-Barradas, C. M., Castelan-Chavez, E. E., & Miliar-Garcia, A. (2015). Expression of NLRP3 inflammasome, cytokines and vascular mediators in the skin of systemic sclerosis patients. *The Israel Medical Association Journal*, *17*(1), 5–10. https://doi.org/10.1002/ART.30568

McCoy, S. S., Reed, T. J., Berthier, C. C., Tsou, P.-S., Liu, J., Gudjonsson, J. E., Khanna, D., & Kahlenberg, J. M. (2017). Scleroderma keratinocytes promote fibroblast activation independent of transforming growth factor beta. *Rheumatology*, *56*(11), 1981. https://doi.org/10.1093/RHEUMATOLOGY/KEX280

Nikitorowicz-Buniak, J., Shiwen, X., Denton, C. P., Abraham, D., & Stratton, R. (2014). Abnormally Differentiating Keratinocytes in the Epidermis of Systemic Sclerosis Patients Show Enhanced Secretion of CCN2 and S100A9. *Journal of Investigative Dermatology*, *134*(11), 2693–2702. https://doi.org/10.1038/JID.2014.253

Okamoto, O., & Fujiwara, S. (2009). Dermatopontin, a Novel Player in the Biology of the Extracellular Matrix. *Connective Tissue Research*, *47*(4), 177–189. https://doi.org/10.1080/03008200600846564

Perbal, B. (2004). CCN proteins: Multifunctional signalling regulators. In *Lancet*. https://doi.org/10.1016/S0140-6736(03)15172-0

Philippeos, C., Telerman, S. B., Oulès, B., Pisco, A. O., Shaw, T. J., Elgueta, R., Lombardi, G., Driskell, R. R., Soldin, M., Lynch, M. D., & Watt, F. M. (2018). Spatial and Single-Cell Transcriptional Profiling Identifies Functionally Distinct Human Dermal Fibroblast Subpopulations. *Journal of Investigative Dermatology*, *138*(4), 825. https://doi.org/10.1016/J.JID.2018.01.016

Quesnel, K., Shi-wen, X., Hutchenreuther, J., Xiao, Y., Liu, S., Peidl, A., Naskar, D., Siqueira, W. L., O'Gorman, D. B., Hinz, B., Stratton, R. J., & Leask, A. (2019). CCN1 expression by fibroblasts is required for bleomycin-induced skin fibrosis. *Matrix Biology Plus*, *3*, 100009. https://doi.org/10.1016/J.MBPLUS.2019.100009

Rastrick, J., & Birrell, M. (2014). The role of the inflammasome in fibrotic respiratory diseases. *Minerva Medica*, *105*(1), 9–23. https://doi.org/10.1002/PATH.4237

Riser, B. L., Barnes, J. L., & Varani, J. (2015). Balanced regulation of the CCN family of matricellular proteins: a novel approach to the prevention and treatment of fibrosis

and cancer. *Journal of Cell Communication and Signaling*, *9*(4), 327. https://doi.org/10.1007/s12079-015-0309-3

Riser, B. L., Najmabadi, F., Garchow, K., Barnes, J. L., Peterson, D. R., & Sukowski, E. J. (2014). Treatment with the Matricellular Protein CCN3 Blocks and/or Reverses Fibrosis Development in Obesity with Diabetic Nephropathy. *American Journal of Pathology*, *184*(11), 2908–2921. https://doi.org/10.1016/j.ajpath.2014.07.009

Riser, B. L., Najmabadi, F., Perbal, B., Rambow, J. A., Riser, M. L., Sukowski, E., Yeger, H., Riser, S. C., & Peterson, D. R. (2010). CCN3/CCN2 regulation and the fibrosis of diabetic renal disease. *Journal of Cell Communication and Signaling*, *4*(1), 39. https://doi.org/10.1007/s12079-010-0085-z

Scholz, G., Martinerie, C., Perbal, B., & Hanafusa, H. (1996). Transcriptional Down Regulation of the nov Proto-Oncogene in Fibroblasts Transformed by p60 v-src. In *Molecular and Cellular Biology*, *16*(2), 481-486. https://journals.asm.org/journal/mcb

Shi-wen, X., Racanelli, M., Ali, A., Simon, A., Quesnel, K., Stratton, R. J., & Leask, A. (2021). Verteporfin inhibits the persistent fibrotic phenotype of lesional scleroderma dermal fibroblasts. *Journal of Cell Communication and Signaling*, *15*(1), 71. https://doi.org/10.1007/S12079-020-00596-X

Solé-Boldo, L., Raddatz, G., Schütz, S., Mallm, J.-P., Rippe, K., Lonsdorf, A. S., Rodríguez-Paredes, M., & Lyko, F. (2020). Single-cell transcriptomes of the human skin reveal age-related loss of fibroblast priming. *Communications Biology* , *3*(1), 1–12. https://doi.org/10.1038/s42003-020-0922-4

Taki, Z., Gostjeva, E., Thilly, W., Yaseen, B., Lopez, H., Mirza, M., Hassuji, Z., Vigneswaran, S., Abdi, B. A., Hart, A., Arumalla, N., Thomas, G., Denton, C. P., Suleman, Y., Liu, H., Venturini, C., O'Reilly, S., Xu, S., & Stratton, R. (2020). Pathogenic Activation of Mesenchymal Stem Cells Is Induced by the Disease Microenvironment in Systemic Sclerosis. *Arthritis & Rheumatology*, *72*(8), 1361–1374. https://doi.org/10.1002/ART.41267

Thompson, K., Murphy-Marshman, H., & Leask, A. (2014). ALK5 inhibition blocks TGFβ-induced CCN1 expression in human foreskin fibroblasts. *Journal of Cell Communication and Signaling*, *8*(1), 59. https://doi.org/10.1007/s12079-014-0229-7

Tsang, M., & Leask, A. (2014). CCN2 is required for recruitment of Sox2-expressing cells during cutaneous tissue repair. *Journal of Cell Communication and Signaling*, *9*(4), 341–346. https://doi.org/10.1007/S12079-014-0245-7

Tsang, M., Quesnel, K., Vincent, K., Hutchenreuther, J., Postovit, L.-M., & Leask, A. (2019). Insights into Fibroblast Plasticity: Cellular Communication Network 2 Is Required for Activation of Cancer-Associated Fibroblasts in a Murine Model of Melanoma. *American Journal of Pathology*, *190*(1), 206–221. https://doi.org/10.1016/J.AJPATH.2019.09.006

van der Slot, A. J., Zuurmond, A.-M., Bardoel, A. F. J., Wijmenga, C., Pruijs, H. E. H., Sillence, D. O., Brinckmann, J., Abraham, D. J., Black, C. M., Verzijl, N., DeGroot,

J., Hanemaaijer, R., TeKoppele, J. M., Huizinga, T. W. J., & Bank, R. A. (2003). Identification of PLOD2 as Telopeptide Lysyl Hydroxylase, an Important Enzyme in Fibrosis *. *Journal of Biological Chemistry*, *278*(42), 40967–40972. https://doi.org/10.1074/JBC.M307380200

Varga, J., & Abraham, D. (2007). Systemic sclerosis: a prototypic multisystem fibrotic disorder. *Journal of Clinical Investigation*, *117*(3), 557–567. https://doi.org/10.1172/JCI31139

Yamamoto, T., & Katayama, I. (2011). Vascular Changes in Bleomycin-Induced Scleroderma. *International Journal of Rheumatology*, *2011*, 1–5. https://doi.org/10.1155/2011/270938

Yamamoto, T., Takagawa, S., Katayama, I., Yamazaki, K., Hamazaki, Y., Shinkai, H., & Nishioka, K. (1999). Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. *Journal of Investigative Dermatology*. https://doi.org/10.1046/j.1523-1747.1999.00528.x

Appendices

Appendix A: Publication Permissions

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Peidl, A., Perbal, B., Leask, A. (2019). Yin/Yang expression of CCN family members: Transforming growth factor β 1, via ALK5/FAK/MEK, induced CCN1 and CCN2, yet suppresses CCN3, expression in human dermal fibroblasts. *PLoS One, 14*(6): e0218178.

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Number of figures/tables	1
Will you be translating?	No
Title	CCN3: A novel anti-fibrotic therapy?
Institution name	Western University

Appendix B: Ethics Approvals

Ethics approval for use of animals was granted under the Animal Use Protocol 2010-253 (Below).

Western

AUP Number: 2010-253
PI Name: Leask, Andrew
AUP Title: The Role Of Adhesive Signaling In Fibrogenesis
Approval Date: 12/02/2014

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "The Role Of Adhesive Signaling In Fibrogenesis" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2010-253::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

Curriculum Vitae

Alex Peidl

EDUCATION

Ph.D. Candidate (Physiology and Pharmacology) **2016 – 2021**
Western University, London, Ontario

- Dissertation: CCN3: A novel anti-fibrotic therapy?

Bachelor of Medical Science (Honours Specialization in Medical Science) **2011 – 2016**
Western University, London, Ontario

PUBLICATIONS

Murphy-Marshman H., Quesnel K., Shi-Wen X., Barnfield R., Kelly J., **Peidl A.,** Stratton R.J., Leask A. *2017.* Antioxidants and NOX1/NOX4 inhibition blocks TGFβ-induced CCN2 and α-SMA expression in dermal and gingival fibroblasts. Plos One 12(10): e0186740.

Peidl, A. *2018*. A friend in knee: CCN3 may inhibit osteoarthritis progression. J Cell Commun Signal 12(2): 489-490.

Peidl A., Quesnel K., Perbal B., Leask A. *2019.* Yin/Yang expression of CCN family members: Transforming growth factor beta 1, via ALK5/FAK/MEK, induces CCN1 and CCN2, yet suppresses CCN3, expression in human dermal fibroblasts. Plos One 14(6): e0218178.

Quesnel K., Shi-Wen X., Liu S., **Peidl A.,** Naskar D., Hutchenreuther J., Siqueira W., O'Gorman DB., Hinz B., Stratton RJ., Leask A. *2019.* CCN1 expression by fibroblasts is required for bleomycin-induced skin fibrosis. Matrix Biol Plus 3: 100009.

Nikoloudaki G., Brooks S., **Peidl A.**, Tinney D., Hamilton DW. *2020.* JNK signaling as a key modulator of soft connective tissue physiology, pathology and healing. Int J Mol Sci 21(3): 1015.

PRESENTATIONS AND SCIENTIFIC MEETINGS

Keystone Symposium: Fibrosis and Tissue Repair **February 2020**
Victoria, British Columbia
International CCN Society Meeting **October 2019**
Niagara Falls, Ontario
FASEB - Matricellular Proteins Meeting **August 2019**
Lisbon, Portugal
London Health Research Day **April 2019**
London, Ontario

American Society of Matrix Biology Conference **October 2018**
Las Vegas, Nevada, USA
Schulich Dentistry Research Day **September 2018**
London, Ontario
Canadian Scleroderma Conference **September 2018**
Calgary, Alberta
Canadian Bone and Joint Conference **May 2018**
London, Ontario
Physiology and Pharmacology Research Day **November 2017**
London, Ontario
Canadian Connective Tissue Conference **May 2017**
Montreal, Quebec
Western Bone and Joint Retreat **May 2017**
London, Ontario

RELATED WORK EXPERIENCE

Teaching Assistant – Human Toxicology **2016 – 2020**
Western University, London, Ontario

Teaching Assistant – Stem Cell Biology and Regenerative Medicine **2020**
Western University, London, Ontario

Teaching Assistant – Introduction to Pharmacology **2019**
Western University, London, Ontario

Research Assistant – Leask Lab **2016**
Western University, London, Ontario

SCHOLARSHIPS AND ACADEMIC HONOURS

Ontario Graduate Scholarship **2019**
Poster Presentation Award – Schulich Dentistry Research Day **2018**
Poster Presentation Award – Canadian Scleroderma Conference **2018**
Poster Presentation Award – Canadian Connective Tissue Conference **2017**
Collaborative Training Program in Musculoskeletal Health Research **2016 – 2018**
CMHR Transdisciplinary Bone and Joint Training Award **2016**
Dean's Honour List – Western University **2014 – 2016**
Queen Elizabeth Aiming for the Top Student Scholarship **2011**

CERTIFICATION AND TRAINING

Comprehensive WHMIS **2019**
Basic Mouse Surgery Techniques **2017**
Laboratory Biosafety **2016**
Basic Mouse Handling **2016**