TGF-β1 requires IL-13 to sustain collagen accumulation and increasing tissue strength and stiffness

by

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This dissertation by Caitlin M. Hopkins is accepted in its present form by the Therapeutic Sciences graduate program as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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- Benjamin T. Wilks, Elisabeth B. Evans, Andrew Howes, Caitlin M. Hopkins, Morcos N. Nakhla, Geoffrey Williams, Jeffrey R. Morgan. Quantifying Cell-Derived Changes in Collagen Synthesis, Alignment, and Mechanics in a 3D Connective Tissue Model. *Advanced Science* (9), 2022.
- Sara Ortega-Atienza, Casey Krawic, Lauren Watts, Caitlin McCarthy, Michal W. Luczak, Anatoly Zhitkovich. 20S immunoproteasomes remove formaldehyde-damaged cytoplasmic proteins suppressing caspase-independent cell death. *Scientific Reports* (7) 654, 2017.
- Sara Ortega-Atienza, Blazej Rubis, Caitlin McCarthy, Anatoly Zhitkovich. Formaldehyde is a potent proteotoxic stressor causing rapid HSF1 activation and Lys48-linked polyubiquitination of proteins. *American Journal of Pathology* (11) 2857-2868, 2016.

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Dedication & Acknowledgements

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Doing a PhD is always difficult, but doing the majority of your dissertation experimentation at the peak of a pandemic was a challenging experience, to say the least. In

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It's been said that education starts in the home, and for me, that was absolutely true. My parents always emphasized the importance of education, both formal and informal, and made numerous sacrifices to ensure my sister and I had all the opportunities to attend the best schools and enrichment programs. You gave me the best start in life and have not stopped supporting me no matter what life throws in the way, whether it's with tuition assistance or childcare for a sick baby. None of this would have been possible without you and I am eternally grateful.

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Chapter 1

Introduction & Background

Many chronic inflammatory conditions ultimately end up resulting in fibrosis, an excessive accumulation of connective tissue that is capable of affecting nearly every organ [1]. Fibrotic diseases include but are not limited to pulmonary fibrosis, liver cirrhosis, various types of sclerosis, and cardiovascular diseases [2]; in total, fibrotic processes affecting various organs accounts for 30-45% of deaths in the developed world [3]. Despite the massive global burden associated with these conditions, at this time there are only two approved antifibrotic therapeutics, which have been tailored to treat idiopathic pulmonary fibrosis (IPF) [4]. There is a great difficulty in the development of new drugs in general, where licensed drugs are the end product of a mere 4% of drug development programs [5]. One of the suggested causes is the failure of animal models and current preclinical study methodologies to properly predict drug efficacy in humans [5]. As such, there is a great need to develop more accurate, predictive models to better understand the development of disease and screen potential treatments.

1.1 The Extracellular Matrix

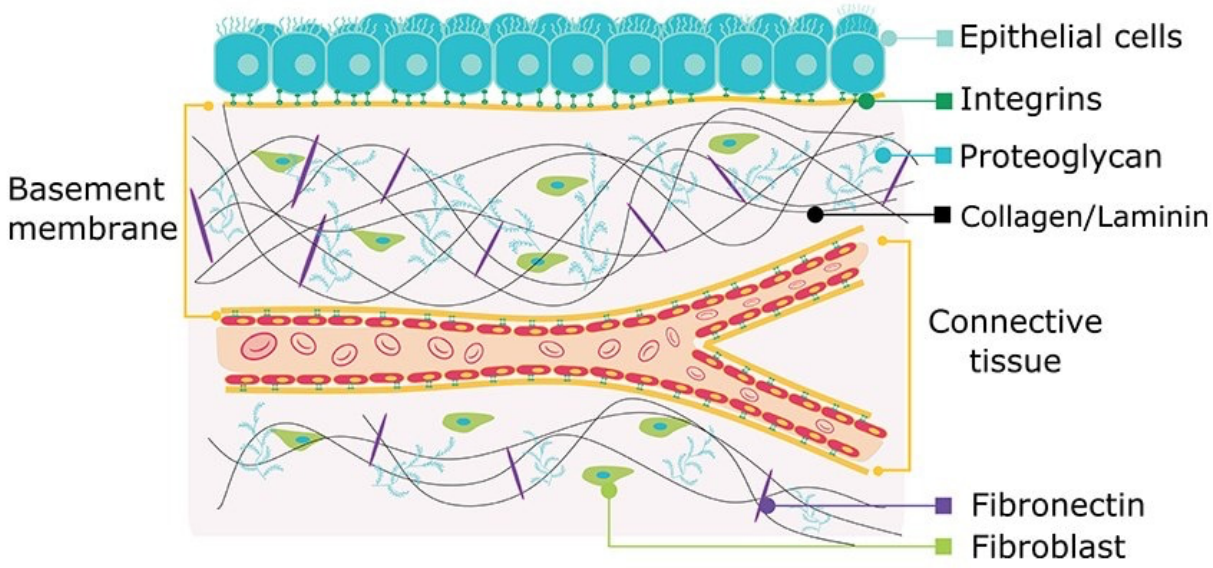
All bodily tissues are composed of both cellular and non-cellular elements, the latter of which come together to form an organized network called the extracellular matrix, or ECM [6]. The ECM is an organized mesh of proteins that acts as a structural support within the body [2], [7]. This “mesh” is also referred to as the core matrisome, and it consists of over 300 different

proteins [8]. It serves as both a physical scaffold and mediator of cues necessary to maintain homeostasis, undergoing persistent remodeling. The two primary categories of molecules in the ECM are proteoglycans, which fill the interstitial space, and fibrous proteins, including collagens, elastins, laminins and fibronectins [9].

The core matrisome is organized into two different and distinct types of ECM. The first surrounds cells, providing structural support, and is called the interstitial connective tissue matrix. The primary proteins in this subtype are collagen I and fibronectin. The second type is the pericellular matrix, which includes the basement membrane (BM), a specialized compact layer of collagen IV, laminins and proteoglycans that separates epithelium and stroma [2], [6], [8]. Overall, while collagens tend to be the most abundant ECM component, the physical and biochemical composition of the ECM is widely heterogeneous as well as tissue-specific, and can vary from organ to organ, within different regions of an organ, or between healthy, aged or diseased tissue [9]. In addition to its structural role, the ECM and its components have a functional role in influencing cell behaviors such as migration, proliferation, adhesion and differentiation [6], [10]. One of the most important functions includes regulation of growth factors and enzymes, which are often released when ECM structural components are cleaved; these factors often play a major role in remodeling the dynamic matrix (**Figure 1.1)**.

The collagen family of proteins are the main structural protein in the ECM, providing tensile strength, and are encoding 43 different genes that make 28 subtypes that are both fibrillar and nonfibrillar [6], [11]. Collagens are characteristically comprised of 3 alpha chains in a helical formation [8], [9]**.** Collagen is mainly produced by fibroblasts and comprises up to 30% of protein in the body [9]. There are 7 categories of collagen, with classification determined by structure and function [12]**.** For example, collagens I, II, III, V, and XI form fibrils, which

Matrix Structure



Matrix Functions

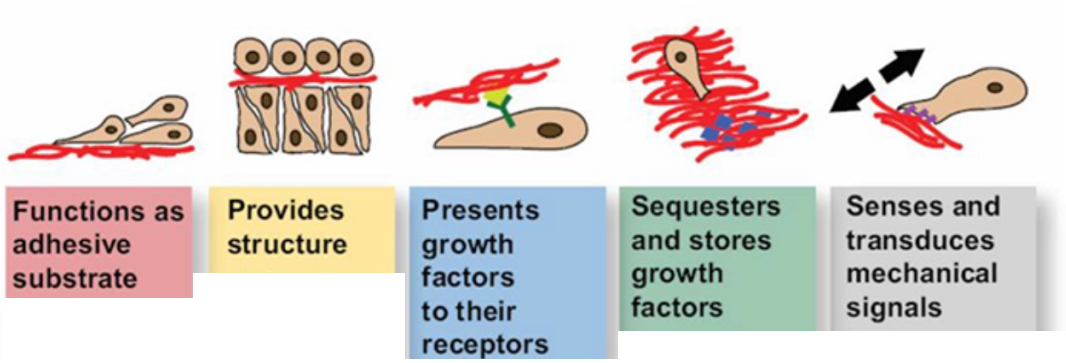


Figure 1.1: The extracellular matrix is a sophisticated and dynamic structure comprised of numerous interconnected components that function to provide structural stability and signaling capabilities to surrounding tissues. Also known as the core matrisome, the matrix is essentially composed of cells including fibroblasts, and associated structural molecules, of which collagen is typically the most abundant, though the precise makeup of the matrix may vary between organ systems and healthy versus diseased tissues. In addition to providing structural support, the matrix is also an adhesive substrate, sequesters various bioactive molecules, and has the ability to sense and transmit mechanical signals through its integrins. Adapted from Millipore Sigma and Rozario et. al. [166], [167]

provide tensile strength and are commonly found in tissues that require resistance to pressure or tensile forces, such as bone, tendon, skin or cartilage. With the exception of cartilage, collagen type I specifically is universally found in the body, and is a major component of scar tissue. Type II is present in cartilage and different parts of the eye. Type III is largely present in vasculature and intestinal organs. Collagen is secreted by various ECM cells including fibroblasts, chondrocytes and osteoblasts [14], [15]. Synthesis of collagen happens at a rate of approximately 40 molecules/cell/second [16]. The most abundantly secreted forms of collagen are collagens I (80%), III (15%), and V (5%) [16]. Fibril formation is an extracellular process facilitated by the cleavage of propeptides by metalloproteinases [17]. Other collagens such as types IV, VIII, and X form networks, including the basement membrane [14], [15]. Fibroblasts use tension on the ECM to organize collagen fibril synthesis and organization**.** Mutations exist that can affect the secretion, assembly, and reorganization of collagen and its networks, and these can lead to a variety of different diseases [6], [9].

Other major ECM components include glycosaminoglycans (GAGs) and glycoproteins; GAGs are interspersed with collagen fibrils and have the ability to bind and sequester growth factors in the ECM, while glycoproteins such as laminins, elastin and fibronectins work to regulate interactions between cells and the ECM [8]. Fibronectin (FN) is critical in the development of vertebrates and is present in the ECM surrounding a variety of cell types [18]. A matrix of FN is produced during tissue remodeling, and is upregulated around tumor vasculature [19]. While collagen is the major structural protein found in the ECM, organs that undergo cyclic stretching such as the lung, skin, and bladder require elastic fibers, which are comprised of elastin and microfibrils such as fibrillins. Elastic fibrils are stable with low turnover, and are important in development; damage is difficult to reverse and leads to improper function [20].

Basement membranes are comprised largely of laminin, which serves as an adhesion site for epithelial cells, and a stabilizing network of collagen IV [21]. Epithelial cells use hemidesmosomes to anchor to the BM, which are formed when laminin interacts with integrins on the cell surface connected to intercellular filaments [22]. Laminins play a key role in organogenesis, as well as facilitate ECM organization and interaction between cells and the matrix [6], [23]. The distribution of their subtypes appears organ specific, suggesting different functions [24]. Their structure and function is dysregulated in a number of disease processes, including tumor progression [25].

Integrins are a family of transmembrane receptors that facilitate interaction between the cytoskeleton and the ECM. They anchor cells to the ECM, and provide mechanotransduction, influencing cell signaling [6], [26]. Bidirectional signaling between the ECM and the cell occurs via integrins, where the ECM signals to intracellular components via outside- in signaling, and intracellular signals are communicated to the ECM via modulation of integrin binding affinity to ECM ligands in inside-out signaling [27]. These receptors bind a variety of ligands, depending on the composition of the heterodimer subunits [28]. Integrins may or may not be constitutively expressed, but rather activated when needed, such as during an inflammatory response [27], [29].

Proteases are important regulators of cellular functions and protein-protein interactions, evidenced by their constituting 2% of genes in the human genome [30]**.** While there are 5 families of proteases, the most important to our context is matrix metalloproteinases, or MMPs [31]. The constant remodeling of the ECM is facilitated by MMPs, which are sub members of the zinc-reliant metzincin family of metallopeptidases. Over 20 MMPs exist, serving different functions, such as the collagenases (types 1, 8, 13) and the gelatinases (types 2 & 9). In concert, all forms are capable of degrading all ECM proteins [32]. They are produced by a large diversity

of cell type, ranging from epithelial cells, to fibroblasts and to leukocytes [14]. MMPs are typically secreted with a pro-domain that is removed during activation by other proteases [32]. At homeostasis, there is little expression of MMPs, but they are upregulated by cytokines and growth factors when remodeling needs to occur [33]. They are also capable of activating latent growth factors, including TGF-β1 [34]. Conversely, tissue inhibitors of metalloproteinases (TIMPs) act opposite MMPs and reversibly inhibit their action [32]. Enzymes such as those in the families of a disintegrin and metalloproteinase (ADAM) or of a disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS) cleave and deposit collagen, respectively [8], [34]. Lysyl oxidases (LOX) promote cross-linking of collagens and elastins [2], [35]. It is important that these enzymes are regulated in order to balance secretion, modification, and degradation of the matrix [2].

In healthy homeostatic tissue, fibroblasts are largely inactive, secreting collagens, elastins, and fibronectins sufficient to maintain the ECM [9], [36]. The healthy ECM is resistant to a number of tensile and compressive forces due to the relaxed network of collagen and elastin, as well as the network of glycosaminoglycans [37]. Further mediators of homeostasis include degrading MMPs and their inhibitors, TIMPs, as well as crosslinking LOX [9]. In aging ECM, MMP degradation is elevated, in concert with reduced protein synthesis [38], [39]. Further, fibroblasts are typically senescent and express high levels of proinflammatory cytokines that result in prolonged inflammation, which destroys elastin and modifies collagen. As a result, ECM is more rigid but less elastic and mechanically weaker than that found in younger tissue [40]. Wound healing, which will be discussed in greater detail, is activated as a result of acute injury to tissue. Vascular damage and clot formation triggers the recruitment of monocytes which differentiate into macrophages that secrete cytokines and growth factors that promote the

migration and proliferation of fibroblasts [41], [42]. These fibroblasts begin to secrete large amounts of collagen and other ECM proteins, increasing mechanical stress and inducing the transformation to myofibroblasts, which further secrete ECM components and are highly contractile. Secreted collagen bundles, particularly if crosslinked, can then greatly affect the strength and stiffness of tissue [42], [43]. In healthy tissue, wound healing processes are limited via feedback mechanisms, but when those mechanisms are compromised or injury is repeated, continued and excessive ECM deposition and remodeling can occur, with little breakdown of matrix, leading to fibrosis [44].

As briefly mentioned in regards to collagen, defects in various ECM components can lead to disease. For example, a defect in collagen I can lead to osteogenesis imperfecta, commonly known as "brittle bone disease" [45]; a defect in laminins can lead to muscular dystrophy, or progressive loss of muscle mass [46]. Loss of precise regulation of the matrix is implicated in numerous common syndromes such as asthma, arthritis, and cardiovascular diseases [7], [47]–[49]. As such, the ECM is very relevant in drug development, due to either direct or indirect effects. In some cases, the effect may not be intended and drugs may unexpectedly bind to the ECM, affecting the efficacy of the drug. ECM components, particularly soluble factors such as proteases or integrins, may be targets due to their roles in various disease states; structural components are not typically considered druggable [10]. One example of a common drug class that modulates the ECM as an off-target effect are NSAIDs, as they function in the inhibition of cyclooxygenase enzymes, which in turn alters the synthesis of collagen and fibronectin [50]. For more examples of molecules that affect the ECM, please refer to **Table 1.1**.

1.2 Wound Healing

A wound is damage to or disruption of normal tissue structure or function; it may be superficial, or within deeper tissues or organs [51]. Wound healing takes place over 4 specific and precisely timed phases that include hemostasis, inflammation, proliferation, and remodeling (**Figure 1.2)**. While overlapping, the four phases of wound healing must occur in a specific order and at the correct intensity for the appropriate duration of time to result in optimal healing. When one of the phases are altered, wound healing can be incomplete or impaired. Complete remodeling results in restoration of tissue architecture and function [52]. Acute wounds are those that follow a normal pattern of wound healing ending in restoration of the tissue in 5-10 days, or within 30 days. Chronic wounds have an altered healing process and do not heal in a timely fashion [51], [53]. Chronic wounds occur in 3-6 million individuals in the US, the vast majority of whom are over the age of 65 and suffering from diabetes or ischemia [54].

Wound healing incorporates both local and migratory cell populations as well as the ECM and inflammatory mediators [55]. While all wounds go through the same general healing process, there are differences in the time to completion, and some tissues such as skeletal tissue, liver, and eye, have their own unique pathways [56]. Different portions of a wound may be in different phases of wound healing at any point [57]. Concurrently to the 4 main stages of wound healing, other critical processes include angiogenesis, reepithelization of the surface of the wound, and maturation of the resultant new matrix, including alignment and crosslinking of the collagen that provides tensile strength to the tissue [52], [58].

Hemostasis occurs immediately after injury. In hemostasis, vascular constriction occurs via contraction of smooth muscle cells within vessels to prevent continued bleeding. Simultaneously, the coagulation cascade is activated, resulting in platelet aggregation and

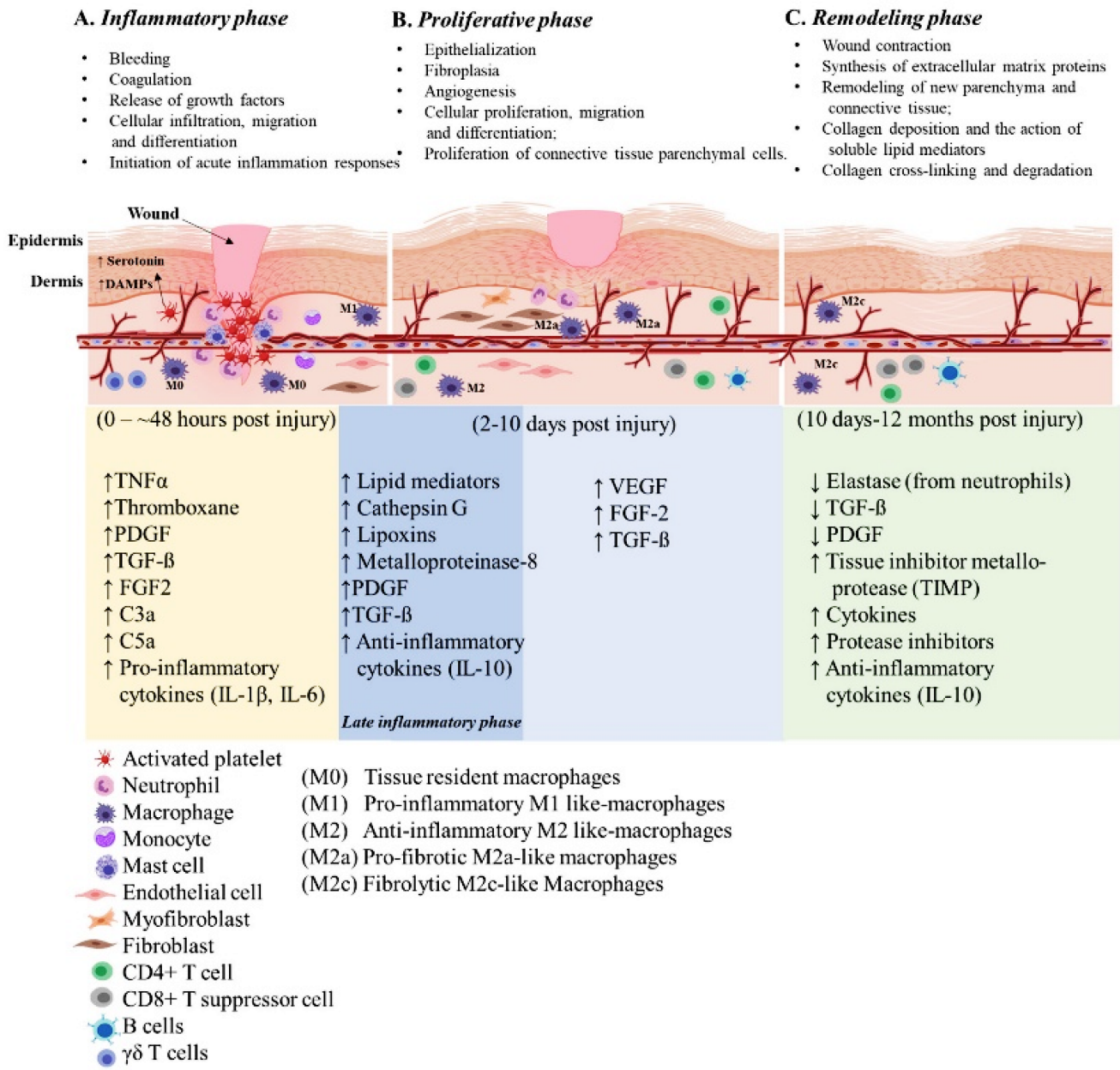


Figure 1.2: Wound healing takes place over the course of four precisely coordinated phases, including coagulation, inflammation, proliferation, and remodeling. After bleeding ceases, various leukocytes infiltrate the wound area removing debris and secreting cytokines that signal for the effector cells, fibroblasts, to begin proliferation and remodeling of the wound. Angiogenesis occurs to allow blood to flow to the area as re-epithelialization takes place. As time goes on, fibroblasts deposit a new collagenous matrix resulting in contraction of the wound. The new matrix is matured through processes of remodeling and crosslinking that results in tissue with stronger tensile strength. Adapted from Muire et. al. [168]

formation of a clot comprised of fibrin, fibronectin, thrombospondin, and vitronectin [59]. Platelet cytoplasm contains a number of pro-inflammatory cytokines and growth factors such as transforming growth factor β (TGF-β1), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) that promote wound healing in their activation of fibroblasts and leukocytes [55].

Inflammation begins shortly after hemostasis and can be categorized into early and late stages [60]. The complement cascade is activated, leading to migration of neutrophils to the wound site, where they phagocytose bacteria to prevent infection [59]. This happens within 24-36 hours, and neutrophils are attracted by chemokines such as TGF-β1. After completion of their role, neutrophils are eliminated via apoptosis [61]. In the late phase, taking place from 48-72 hours post-injury, monocytes arrive and undergo a phenotypic transformation to macrophages. Macrophages continue phagocytosis with a greater lifespan than neutrophils [51], [62]. These macrophages release more cytokines that attract more leukocytes, remove apoptotic cells, and stimulate cell types including fibroblasts and keratinocytes to begin regeneration of the tissue [52]. Macrophages are critical to wound healing such that when they are depleted, inflammation decreases [62]. Conversely, if macrophages fail to clear via apoptosis after acute repair, excessive repair can occur, leading to fibrosis [63]. As part of the last portion of the inflammatory stage, chemokines produced by macrophages recruit T- lymphocytes to the damaged tissue [62]. T-lymphocytes then appear, though their specific role in wound healing is not largely understood and may depend on whether they are CD4+ or CD8+ [64].

Proliferation occurs within days of the initial injury and can take place for up to two weeks. Key elements of the proliferative phase include the reepithelization of the wound surface, angiogenesis, and collagen deposition [51], [52]. Inflammatory cells that release factors such as

TGF-β1 or PDGF attract fibroblasts and myofibroblasts, which then proliferate and produce matrix components such as proteoglycans, fibronectin, and procollagens [65]. Fibroblasts may later differentiate to myofibroblasts, which attach to components such as collagen and fibronectin and begin to contract to facilitate wound closure. Fibroblasts are eliminated by apoptosis once their tasks are complete [66]. During all phases of the wound healing process, angiogenesis is concurrently occurring, where blood vessels from the margin of the wound sprout new capillaries to perfuse the site of the injury [51], [67].

Wound remodeling encompasses scar formation, and may take place over the course of up to a year, depending on the severity of the wound. This remodeling is a tightly regulated process with the goal of maintaining a balance between matrix synthesis and degradation, where equilibrium is typically achieved within 3 weeks [68]. Collagen degradation is mediated by leukocyte-secreted MMPs. Initially active, their activity eventually decreases to allow for matrix accumulation [51]. As the matrix matures, bundles of collagen increase their diameter, become more aligned, and cross-link, resulting in increased tensile strength. As the wound returns to a state of normality, cellularity decreases and capillary growth ceases [68].

Factors that affect the efficacy of the wound healing process include age, sex, hormones, oxygenation to the wound, medical conditions such as diabetes, general nutrition and smoking status [52]. Prolonged hypoxia delays the process of wound healing and can over-amplify the inflammatory response [69], [70]. During wound healing, the removal of bacteria and other contaminating microorganisms is key, and if not completed can extend inflammation past its normal duration. This extended inflammation can result in upregulation of ECM-degrading MMPs [54], [71]. Aging can result in a delay of the normal wound healing mechanisms and an altered inflammatory response [72]. Interestingly, compromised wound healing is more common

in males than in females, likely due to the protective effects of estrogen in comparison to the negative effect of androgens on these processes [73]. Finally, some commonly used medications including anti-inflammatories, steroids, and chemotherapeutics can affect clot formation, the function of platelets, cell proliferation, or general inflammation such that wound healing is negatively affected [52].

1.3 Fibrosis

Fibrosis, or the excessive accumulation of ECM, is a characteristic of most chronic inflammatory diseases [1]. Fibrosis also occurs when ECM production occurs without balanced degradation [8]. ECM remodeling is important for homeostasis and processes such as wound healing, but it is problematic when it becomes either excessive or uncontrolled. On a molecular level, there are four main stages to the fibrotic response- an injury occurs that elicits an inflammatory response; effector cells are activated; the ECM is excessively remodeled; and there is an imbalance between ECM deposition and resorption (**Figure 1.3)**. As a result of these four stages acting in concert with one another, fibrosis and subsequent organ failure can occur [74].

Fibrosis is commonly triggered by inflammation, either acute or chronic, and damaged endothelial or epithelial cells utilize chemotactic factors to recruit inflammatory cells such as macrophages, neutrophils, or eosinophils. These immune cells also produce inflammatory growth factors and cytokines that amplify cell injury and activate fibroblasts or myofibroblasts, the main producers of ECM in fibrosis [1], [16], [67]. Each mediator functions in a different manner, but generally contributes to the fibrotic process. For example, as one of the most potent mediators of fibrosis, TGF-β1, causes the translocation of the SMAD 2/3 complex to the nucleus, where it upregulates ECM genes such as collagen [75]. The process of fibrosis is very

similar to that of wound healing, with just a few key differences; in the process of wound healing, signaling is regulated and the overall process is transient, whereas in fibrosis, cytokine activation is chronic and deposition of ECM occurs in a manner that is more excessive than that which is required for repair of an injury, and actually leads to tissue deformation and impaired function rather than restoration [76].

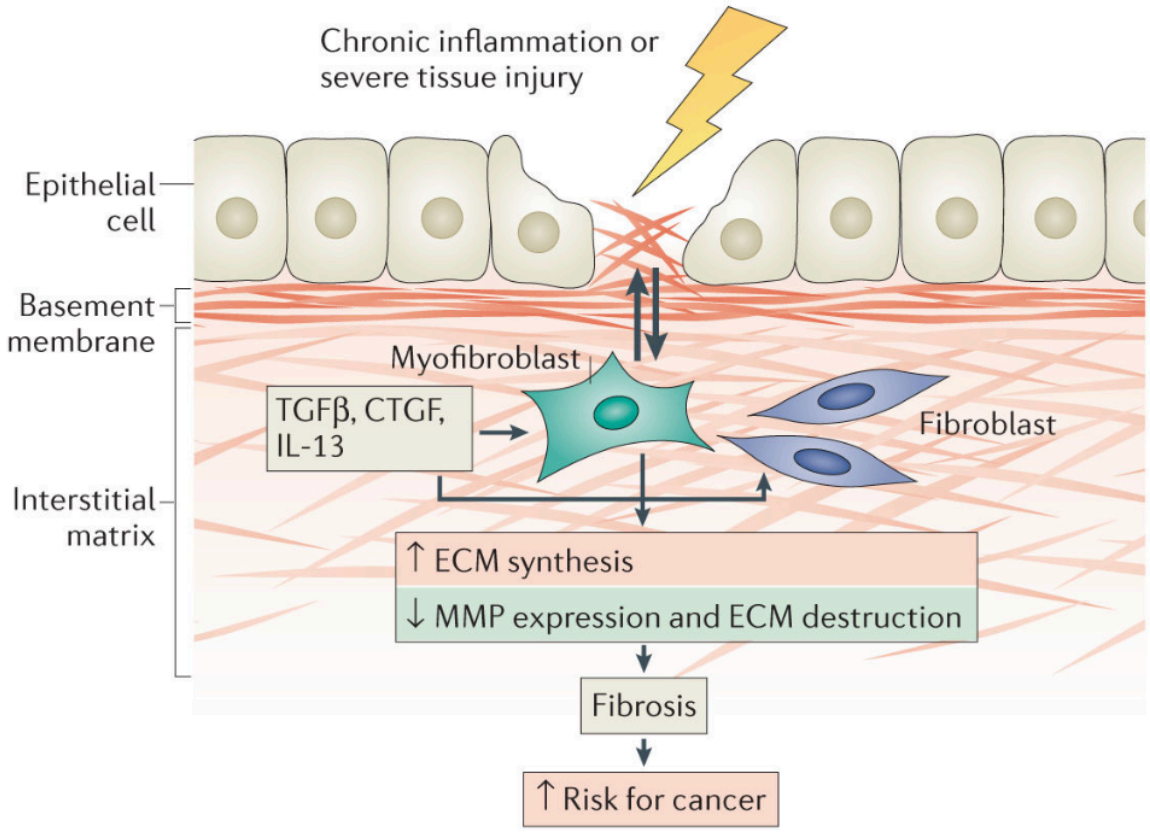


Figure 1.3. Fibrosis, which has the potential to increase the risk of cancer, is a result of imbalanced ECM synthesis due to tissue injury. When tissue injury takes place, wound healing responses are activated but become dysregulated. Cytokines such as IL-13 and TGF-β1 stimulate fibroblasts to differentiate into active, matrix-depositing myofibroblasts. In fibrotic processes, matrix synthesis outweighs matrix degradation, resulting in excessive collagen accumulation causing increased tissue stiffness and decreased elasticity. Adapted from Bonnans et. al. [3]

Alterations in matrix biomechanics is a common occurrence in fibrotic diseases. As a result of increased collagen deposition, tissue stiffness typically increases while elasticity decreases, which can lead to mechanical stress, further exacerbating tissue injury and perpetuating the activation of alpha- smooth muscle actin (αSMA) expressing myofibroblasts [77]. Further amplifying the effect of the increased amount of collagen deposition in fibrosis is limited ECM breakdown; in pulmonary fibrosis, as an example, MMPs are downregulated while TIMPs are overexpressed, leading to a shift in balance favoring matrix deposition over resorption [78]. Another mechanism through which ECM stiffening can occur is through increased collagen fibril crosslinking, which is mediated through LOX. The expression and activity of LOX is elevated when there is an increased deposition of collagen, such as there is in fibrosis [2], [79]. One consequence of this increased stiffening is that profibrotic TGF-β1 bound to the ECM may be more readily available [78]. Thus, a goal in repair of fibrotic tissue is the restoration of mechanical properties of the ECM [80].

Organ fibrosis is fairly plastic, and has the potential to regress if the profibrotic stimulus is removed, or an antifibrotic therapy is administered, though the degree of improvement may vary from organ to organ. When fibrosis is able to regress, clinical outcomes tend to improve. Realistically, it may be more appropriate to aim to slow the progression of disease, rather than resolve the condition completely [74]. Because the initial uncontrolled wound healing response is largely universal between tissue types, genome-wide association studies (GWAS) have demonstrated significant overlap and conservation of gene pathways susceptible to the development of fibrotic diseases in different organ systems [81]; this may be useful when considering development of therapeutic targets that are applicable to several different types of fibrosis (**Table 1.2)**. This is nuanced, however, because targeting the inhibition of pleiotropic

cytokines and growth factors can have unintended effects on other cell types and tissues; furthermore, some profibrotic cytokines also have antifibrotic and regenerative properties dependent on context, additionally confounding choice of therapeutic target [82].

1.4 Fibroblasts

As previously discussed, fibroblasts are the primary connective tissue cell type and source of ECM. Fibroblasts are a mesoderm-derived dynamic cell type that largely function in production and remodeling of ECM components, particularly within the context of tissue repair or regeneration. They assist in maintaining the shape of the tissue they inhabit, but also secrete various different bioactive mediators that signal for tissue repair. Through these mediators, activated fibroblasts influence neighboring cell types [83]. Aside from matrix production, fibroblasts play roles in angiogenesis and inflammation [84]. They are also capable of facilitating post-translational modification of the ECM, including cleaving of procollagen, proteolytic degradation, and remodeling such as crosslinking [63].

During normal wound healing processes, inactive fibroblasts transform their phenotype into myofibroblasts, an active cell type that is highly contractile and proliferative, and produces matrix at an accelerated rate [63], [85]. Fibroblast activation occurs via 4 major mechanisms- cells are stimulated by either paracrine or autocrine growth factors, through contact with other cells, through matrix integrins, or via environmental conditions such as hypoxia [86]. Resident tissue fibroblasts are commonly activated in a paracrine fashion via cytokines produced by immune cells [87] (**Figure 1.4**). However, fibroblasts themselves also secrete a series of cytokines and reactive oxygen species (ROS) that act in a paracrine fashion to in turn activate and mobilize macrophages and other immune cells. Paracrine signals from immune cells that act

on fibroblasts to induce inflammation and induction of fibrosis include PDGF, IL-13 and leukotrienes. Fibroblasts are also capable of producing autocrine agents such as TGF-β1, IL-6, and IL-1β [84]. Inhibition of the interaction of these profibrotic cytokines with their receptors has been a therapeutic strategy in the treatment of fibrotic disease [88].

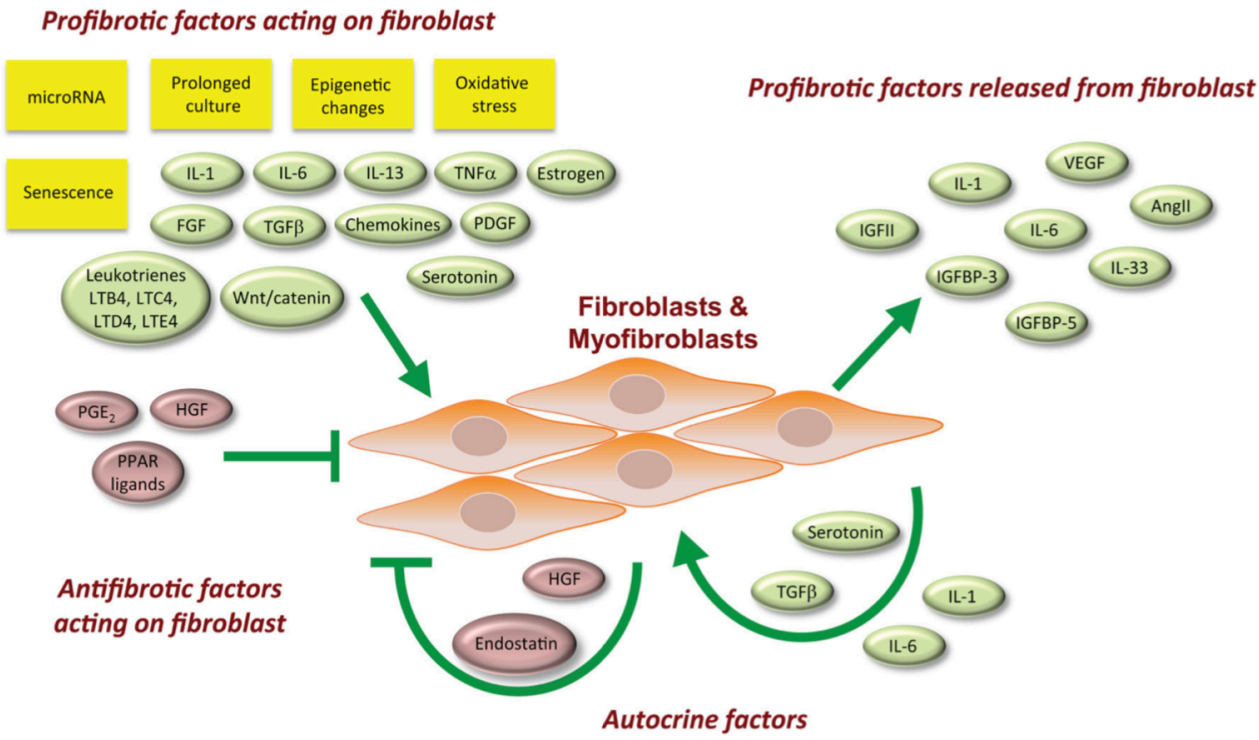


Figure 1.4: Communication to and from fibroblasts occurs via a number of cytokines and growth factors. These cytokines may be secreted by immune cells which then act in a paracrine fashion on fibroblasts, or from fibroblasts themselves which can act in a paracrine manner on other cells, or in an autocrine manner on themselves. For example, profibrotic factors such as interleukins, leukotrienes, and TGF-β1 act to differentiate fibroblasts into their more active myofibroblast phenotype. Conversely, paracrine signals from factors such as PPAR ligands have an antifibrotic effect on fibroblasts. Interestingly, while TGF-β1 may be secreted by immune cells, fibroblasts are also capable of secreting it, where it then has the ability to act in an autocrine fashion. Adapted from Kendall & Feghali- Bostwick [84].

Interestingly, myofibroblasts can derive not only from fibroblasts, but can be generated from a variety of cell sources, including but not limited to smooth muscle cells, fibrocytes, pericytes, epithelial cells, and endothelial cells [89]. Myofibroblasts express αSMA, and this biomarker expression serves as a benchmark for fibroblast activation, in addition to increased proliferation and ECM production. Myofibroblasts behave in a manner similar to both fibroblasts and smooth muscle cells [87], [90]. In high numbers, these myofibroblasts can produce an excessive amount of ECM. Within the context of fibrosis, the number of myofibroblasts present often correlates to the severity of the disease. There is evidence that changes in metabolism can affect the activation of fibroblasts and disease progression. When tissue injury persists or becomes chronic, fibroblasts need to increase their energy to continue to proliferate and synthesize proteins, requiring metabolic adaptations, particularly involving ATP production and oxidative phosphorylation. As a result, glycolysis is often increased during fibroblast activation and resulting fibrosis [91].

When myofibroblast activation is prolonged, worsening fibrosis can affect tissue architecture and function [87]. One such cell type that appears to be heavily involved in fibroblast activation are resident tissue mast cells, which are also implicated in multi-systemic inflammatory conditions resulting in fibrosis [92]. As such, it is important that activation be precisely controlled. Of note, however, is that removal of leukocytes such as monocytes and macrophages after a tissue is injured hinders the ability of myofibroblasts to accumulate, produce collagen, and facilitate angiogenesis, suggesting a critical and complex interplay between the immune system and fibroblasts for proper wound healing [87], [93].

1.5 TGF-β

Perhaps one of the most critical growth factors associated with the ECM within our context is transforming growth factor beta (TGF-β). The TGF-β superfamily of cytokines encompasses over 30 members, including TGF-β in all its isoforms, as well as activins and bone morphogenetic proteins (BMPs) [94]. TGF-β1 is a 25 kDa homodimer with many functions, including synthesis of matrix proteins, that is sequestered in a latent form attached to the ECM and needs to be released to be activated and bind with its receptor [10], [95]. These cytokines bind to serine/threonine kinase receptors and signal via SMAD proteins [94] (**Figure 1.5)**.

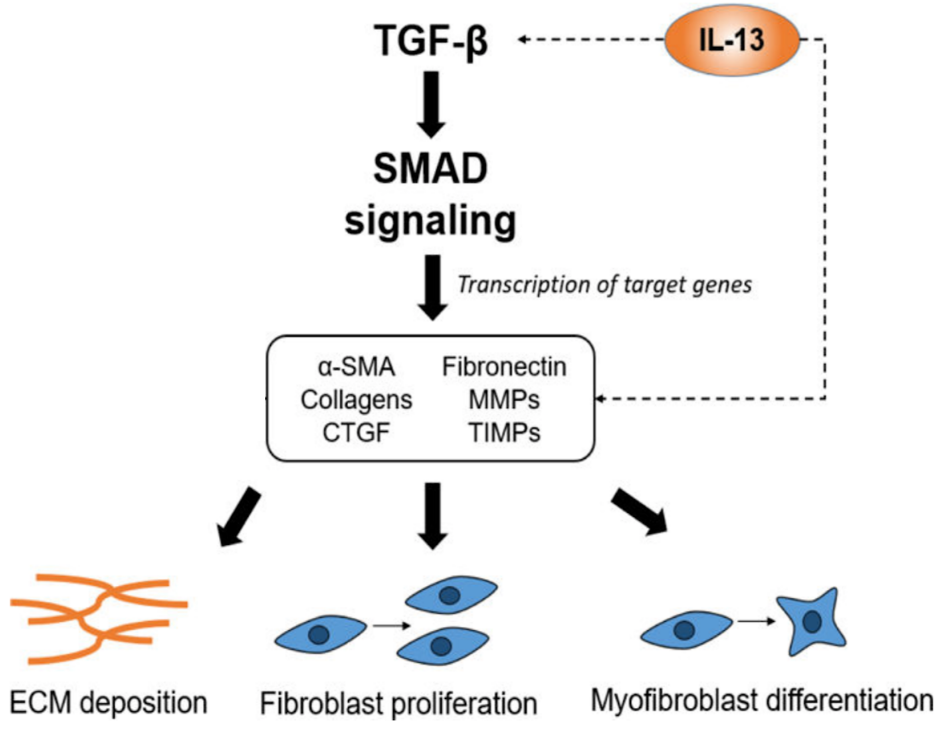


Figure 1.5: TGF-β1 works to elicit its effects through the SMAD signaling pathway. When TGF-β1 is working in a profibrotic manner, it signals through SMAD to affect transcription of target genes such as collagens and matrix remodeling enzymes that ultimately lead to increased matrix deposition and proliferation and differentiation of fibroblasts to myofibroblasts. Interestingly, IL-13 has the ability to stimulate TGF-β1 production and function, exacerbating these effects. Adapted from Nguyen et. al [106].

Briefly, latent TGF-β1 binding proteins (LTBPs) bind to a latency associated peptide (LAP) associated with TGF-β1 to form the large latent complex (LLC), which is bound to the ECM via the structural protein fibrillin, which plays a role in elasticity [35]. TGF-β1 is activated from its latent form in a number of ways, including by proteases that degrade the LAP or LTBP, or by integrins that initiate mechanical pulling, thus altering the structural conformation of the complex, allowing for TGF-β1's release [95], [96]. Fibroblasts also have the ability to activate latent TGF-β1 via metabolomic reprogramming; active fibroblasts increase glycolysis which increases lactate, decreasing the pH of the extracellular environment and activating sequestered TGF-β1 [96].

As mentioned, TGF-β1 and its superfamily plays a number of roles in the body. Functions include but are not limited to regulation of growth, tissue differentiation, and apoptosis [97]. Activins and BMPs are thought to play a greater role during embryogenesis [98], [99], while TGF-β1 influences later development and adulthood [100], [101]. TGF-β1 is a growth inhibitor in many cell types, and also functions as a fibrogenic factor in many tissues [94]. In addition to modulating cell proliferation, TGF-β1 also influences cell interactions with the ECM, and aids in the synthesis of ECM components. TGF-β1 is also capable of increasing integrin expression, which mediates interactions between cells and components of the ECM; this often happens in conjunction with inhibition of matrix degrading proteases, contributing to fibrosis [102]. Depending on the cellular source, TGF-β1 has the ability to be either anti-inflammatory or profibrotic. When secreted by macrophages, TGF-β1 tends to act in a profibrotic manner, but when secreted by CD4+ regulatory T cells, it acts as an anti-inflammatory signal [1]. This lends to the idea that TGF-β1 function is multifactorial and dependent on both context and environment, and has the ability to act as a "switch", where it facilitates the activation of a

process in stasis, but will also cause the same process to discontinue at the appropriate time [103].

Crosstalk with other pathways including MAP kinase and JAK/STAT may also influence TGF-β1 signaling [104]. These cytokines may be positively or negatively regulated, amplifying signals to instigate biological activity, or limiting them to end downstream activity. Negative regulation occurs at a number of locations, including at the nuclear, cytoplasmic, membrane, and extracellular levels [94]. Active TGF-β1 can be degraded by proteases and elastases that are released at the site of inflammatory responses [105]. TGF-β1 also binds to matrix components, which serve as a reservoir for their store or release [102]. Most cells secrete TGF-β1 and/or have TGF-β1 receptors, indicating occurrence of both autocrine and paracrine signaling [97]. Different factors modulate TGF-β1 expression levels, including substrate stiffness; a stiffer substrate such as cell culture plastic results in higher expression, whereas in contrast, cells on a matrix or basement membrane downregulate cytokine expression. As a result, synthesis of TGF-β1 is more restricted *in vivo* than it is *in vitro* or on tissue culture plastic [102].

1.6 IL-13

IL-13 has been identified in several studies as being the dominant cytokine in fibrosis [62]. IL-13 is produced by mast cells, eosinophils, T helper type 1& 2 cells, and type 2 innate lymphoid cells (ILC2s), and binds to activate the JAK/STAT6 signaling pathway [84], [96], [106], [107] (**Figure 1.6)**. As a key mediator of inflammation, IL-13 has been shown to play a large role in the development of allergies and asthma [108]. Furthermore, asthma and

schistosomiasis have demonstrated IL-13 as a crucial mediator of fibrosis, and thus regulator of the ECM, within a disease context [109].

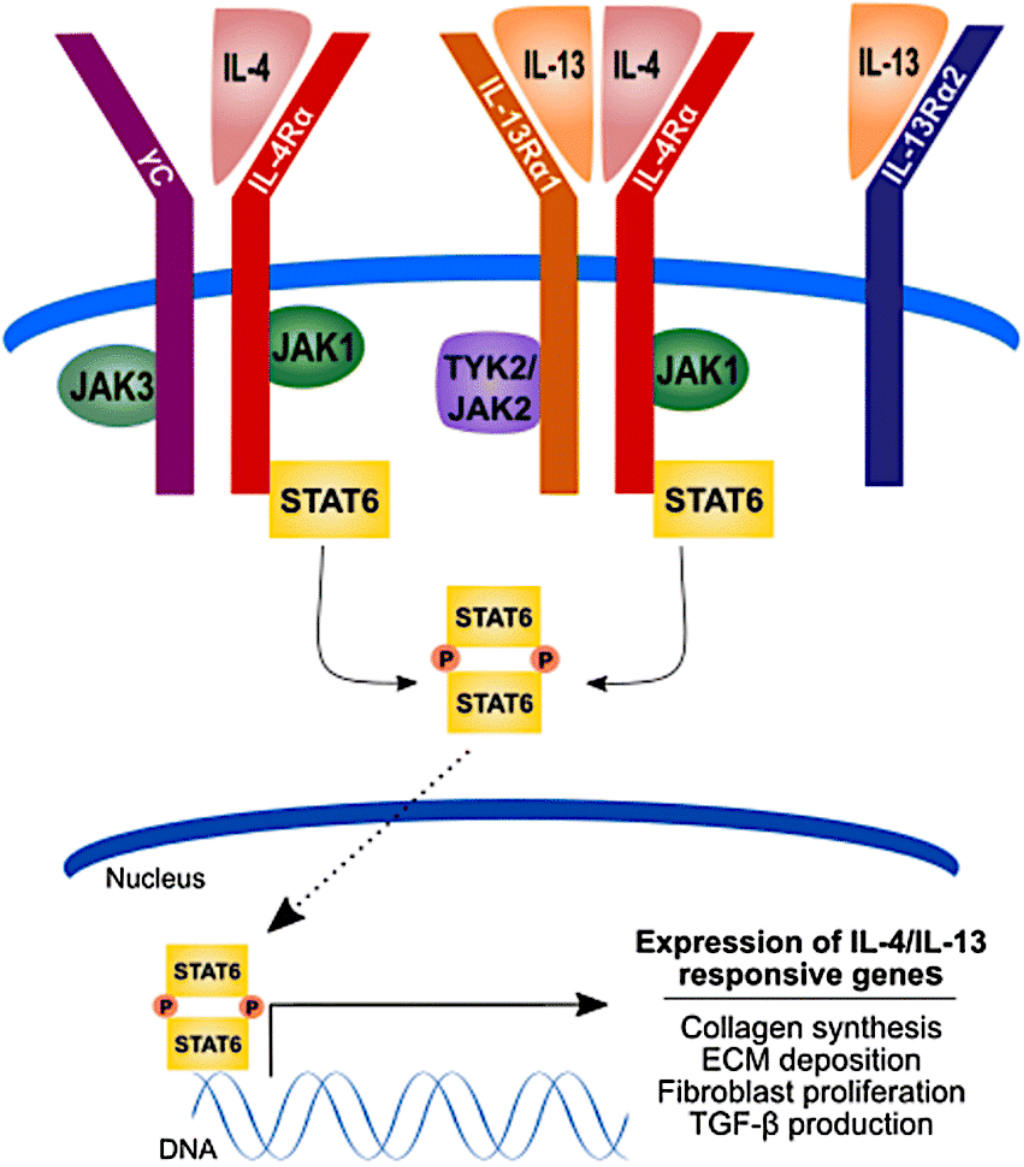


Figure 1.6: IL-13 elicits its effects by working through the JAK/STAT signaling pathway. IL-13 has a structurally similar sibling cytokine, IL-4, with which it forms a heterodimeric receptor complex to signal through, affecting the transcription of genes that lead to matrix synthesis and deposition, fibroblast proliferation, and production of TGF-β1. IL-13 also has a secondary receptor, often referred to as a "decoy" receptor, that sequesters the ligand and has no well-characterized downstream signaling capabilities. Adapted from Nguyen et. al [106].

IL-13 is similar to IL-4, another cytokine from the interleukin family. IL- 4 and IL-13 are structurally similar type 2 secreted glycoprotein cytokines that work within the context of type 2 inflammatory responses. These responses are triggered by multicellular organisms, pathogens, and allergens [110]. While structurally similar, there is only 25% overlap in amino acid sequences; IL-4 is divergent among species, while IL-13 is more conserved [111]. Both genes are located on human chromosome 5 [112]. In many diseases, IL-13 is produced at a much higher abundance than IL-4 (>10 fold greater), possibly making it more effective in mediating fibrosis [113], [114].

These cytokines signal via engagement of heterodimeric receptor complexes [115]. A receptor complex comprised of both IL-4Rα and IL-13Rα1 chains is capable of binding both ligands and is expressed on a variety of leukocytes, fibroblasts, smooth muscle cells, and endothelial cells [108], [109]. IL-4 and IL-13 receptors require activated JAKs for signal transduction, and STAT6 is major transcription factor pathway utilized by the cytokines for signaling [108], [110], [116]. An alternative receptor for IL-13, IL-13Rα2, seemingly inhibits its activity [114]. IL-13Rα2 has a short cytoplasmic tail with no signaling motifs, supporting the concept of a decoy receptor, as the primary function appears to be limiting the effect of IL-13 [108], [109], [117]. Interestingly, IL-13Rα2 binds IL-13 with a much higher affinity (K_D = 250 pmol/L) than the IL-4/IL-13 receptor complex (K_D = 2-10 nmol/L) [114], [117], [118]. Furthermore, studies have shown that when IL-13Rα2 is negated, IL-13 signaling is enhanced, increasing fibrosis; concurrently, ligand abundance decreases, indicating the receptor itself is influential in determining collagen deposition [114], [119].

Both IL-4 and IL-13 have many functions including playing roles in inflammation, allergic reactions, and fibrosis, as well as regulation of production of immunoglobulins [110],

[120]. Many functions of wound healing are reliant on IL-4/IL-13, as they help drive production of TGF-β1, collagen deposition, and augment the profibrotic activity of TGF-β1 itself. Their activation and polarization of macrophages is necessary for attenuating inflammation and promoting remodeling [110], [121]. Both type 1 (ex. IFNγ and IL-12) and 2 cytokines (ex IL-4/IL-13) participate in inflammation, but have opposite roles in fibrosis, where type 2 cytokines directly stimulate fibroblasts *in vitro* to produce collagen [107], [109], [119].

IL-13 specifically is an anti-inflammatory cytokine that becomes a driver of fibrosis when normal immune responses during the process of wound healing are overactive or dysregulated [106]. IL-13 is capable of activating fibroblasts, causing their proliferation, and stimulating them to release collagen. This activation of fibroblasts via IL-13 can occur both directly via receptor pathways or indirectly via activation of other messengers [122]. *In vivo*, IL-13 assists in the development of fibrocytes, or hematopoietic cells that are capable of producing collagen. In addition to synthesis of collagen, fibrocytes may also secrete soluble profibrotic factors such as connective tissue growth factor (CTGF) that activates fibroblasts to myofibroblasts [123]. As IL-13 mediates collagen production in fibroblasts, the effect on fibrosis may be direct, in addition to its indirect influence via activation of other mediators, or aggravating the cytokine responses [107], [109], [119]. Macrophages and dendritic cells may also be effectors of IL-13 in inducing fibrosis and inflammation [109], [124].

1.7 Rationale for combination of the factors

Some additional evidence suggests that IL-13 activity is capable of being modulated by TGF-β1, and that IL-13 may also in turn activate TGF-β1, implying cross-talk between the two pathways [106], [125]. Some evidence suggests that fibrosis occurs through pathways other than

SMAD, stating that collagen deposition can occur independently of TGF-β1, as a result of stimuli with cytokines such as IL-13 alone [126]. Some sources also show that cytokines like IL-13 can activate macrophages to produce TGF-β1 in its latent form, and that proteins that cleave the LAP rendering TGF-β1 active may also be upregulated [127]. Of importance is whether or not IL-13's "decoy" receptor truly serves as a decoy, or whether it has signaling abilities; in reality, its function may be environmentally dependent. For example, some evidence suggests signaling via IL-13Rα2 may help promote TGF-β1- driven fibrosis, utilizing a pathway other than JAK/STAT6 [128], [129]. This idea stems from studies that have shown signaling function from the decoy receptor in activating the production of TGF-β1 [128], [130] (**Figure 1.7**). Specifically, when IL-13 signals through the IL-13Rα2, it induces a transcription factor called AP-1 that activates the TGF-β1 promoter [128], [131]. This "decoy" receptor has been said to be activated by a combination of IL-13 itself and TNF-α [128]. However, other evidence suggests that the decoy receptor can in fact act as the name implies, via sequestration of the ligand it has such a high affinity for, thus preventing its downstream effects [109], [132], [133].

Groups have looked at the effect of both TGF-β1 and IL-13 on fibroblasts *in vitro*, with results that prompt more inquiry. Murray *et al* found that non-fibrotic fibroblasts responded to TGF-β1, but not to IL-13 [134]. However, studies examining human airway fibroblasts found that IL-13 augments TGF-β1's induction of TIMP1, and that treatment with the combination of TGF-β1 and IL-13 also increased fibroblast production of eotaxin-1, which leads to increases in collagen I [135]. Another study by Zhou *et al* determined that TIMP1 expression in airway fibroblasts is reliant upon SMAD2/3 phosphorylation, and that IL-13 augments this action in response to TGF-β1 [136].

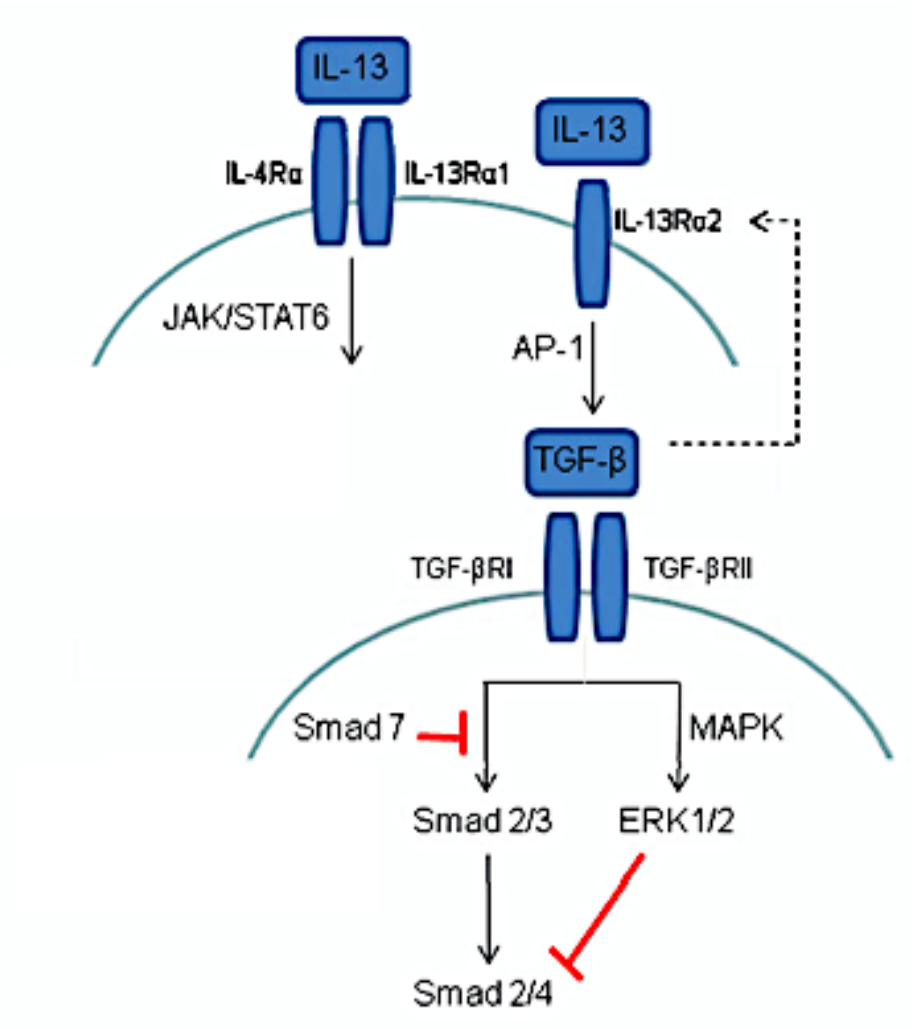


Figure 1.7: Some reports indicate that IL-13's "decoy" receptor may in fact be capable of participating in some signaling function. When IL-13 works through its heterodimeric receptor with IL-4, the JAK/STAT pathway is activated, eliciting a number of profibrotic downstream effects. When IL-13 binds to its "decoy" receptor, it is unclear whether there is a functional signaling domain. Recent evidence indicates that rather than serving as a nonfunctional decoy receptor, binding to the IL13Rα2 mediates signal transduction through the AP-1 pathway, one effect of which is induction of TGF- β. Adapted from Hold et. al [169].

In vivo studies also give compelling evidence for interaction between TGF-β1 and IL-13; for example, studies by Kaviratne *et al* show IL-13 KO mice demonstrated a reversal of fibrosis, even when exposed to TGF-β1. Similarly, TGF-β KO mice experienced a 20- to 50- fold

increase in collagen I and III expression when treated with IL-13 [137]. Interestingly, in bleomycin lung models, inhibition of IL-13Rα2 led to decreased levels of TGF-β1 and subsequently attenuated levels of collagen deposition [128]. Together, these studies suggest both an IL-13 dependent, TGF-β1 independent pathway for fibrosis, as well as pathway overlap and cross-talk that is worthy of further investigation.

1.8 *In vivo* fibrosis models

In studying fibrosis in animals, the most commonly used model is that in which fibrosis is induced by bleomycin (BLM) treatment, typically in mice. Bleomycin is a chemotherapeutic antibiotic that is thought to work by causing DNA breaks and inducing apoptosis, fibroblast activation, and ECM deposition [138]. While it is considered the most accurate interstitial pulmonary fibrosis (IPF) model in terms of its molecular signature, there are significant shortcomings, including how fast the condition develops upon treatment, and that it typically resolves itself [138]–[140]. However, because it is currently considered the most clinically relevant murine fibrosis model, most preclinical models for potential antifibrotic therapeutics utilize BLM-induced injury models. Endpoints for these studies typically include simple histology and measurements of collagen deposition, which is not necessarily medically useful in that it does not give information on quality of lung function. Thus, additional metrics that are recommended for assessment of lung fibrosis include evaluation of apoptosis, examination of respiratory mechanics, and analysis of inflammatory and fibrotic biomarkers. It is further recommended that BLM murine models are used as first line screening tools, followed by studies in an additional 2-3 animal models, which is both expensive and time consuming [138], [141], [142].

In vivo may widely be considered the best way to look at interactions between cells and the ECM, but there are certainly a number of imperfections. Specifically, many *in vivo* studies look solely at how cells may be manipulated, rather than how ECM can be tunable either biochemically or biomechanically [2]. *In vivo* models in general by nature often do not allow for precise manipulation of the ECM, and typically only cellular aspects can only be indirectly controlled. Further, failings of potential therapeutics in humans after undergoing animal testing have demonstrated significant differences between human and animal physiology [143]–[145]. For these reasons, there is a consensus that animal models do not entirely recapitulate the disease process occurring in a number of fibrotic diseases (**Table 1.3**). However, they can be useful in identifying pathways that lead to or molecules that induce fibrosis, such as TGF-β1 pathways or MMPs [146]. As such, human 3D *in-vitro* models are being developed to help further understand disease pathologies and as a tool for high through-put (HTP) drug screening [147].

1.9 Considerations for *in vitro* models

Countless groups have studied fibroblasts *in vitro*, both in a healthy and fibrotic context. Members of the Morgan lab have shown that scaffold-free micromolds allow for cell self-assembly and the control of ECM synthesis in various geometries [148]–[151]. Choice of cell determines matrix composition. Fibroblasts are capable of forming stable ring tissues and secrete their own highly aligned, collagen-rich matrix. The complexity of these matrices have the potential to recapitulate the composition and architecture of *in vivo* ECM, and have mechanical properties within range of natural tissues [149], [152].

To further regulate *in vitro* cultures, cell culture substrates are tunable and can be created to have various elastic moduli recapitulative of different tissue elasticities; when cells are

cultured on these substrates matching their tissue of origin, they take on phenotypes that are more *in vivo*- like than if the cells were grown on plastic substrates [80], [153]. ECM can also be modulated *in vitro*; cell remodeling creates anisotropy, where the elastic modulus will be higher in regions of tissue where pulling occurs. By prestraining the ECM in biomechanical tissue culture experiments, increased activation of latent TGF-β1 sequestered in the matrix occurs, allowing this now-available TGF-β1 to subsequently contribute to activation of fibroblasts [80], [154]. To strengthen *in vitro* ECM through increased stiffness, LOX can be utilized to stimulate cross-linking [2].

As discussed, proinflammatory growth factors and cytokines are critical in the development of a fibrotic state, and this has been demonstrated *in vitro*. Mia *et al* determined that when exposed to TGF-β1, both dermal and lung fibroblasts show increased expression of αSMA, and upregulation of *COL1A1*; additionally, levels of LOX increase 1.5-2 fold when compared to control, nontreated fibroblasts [155]. Exposure to IL-13 stimulates fibroblasts as well as increases αSMA expression and collagen deposition while inhibiting MMPs and enhancing TIMPs [107], [156]. PDGF has been demonstrated to increase contractility of fibroblasts, while also facilitating their differentiation into myofibroblasts [157]. *In vitro*, fibroblasts cotreated with CTGF and TGF-β1 have shown increased proliferation and synthesis of collagen I and fibronectin [158], [159].

When assessing the quality of an *in vitro* model for suitability to replicate changes in the ECM many factors must be considered, including relevance to *in vivo* physiology and quality of endpoints. It is important to be able to monitor changes in a temporal fashion, compared to solely as an endpoint. This can be done by taking tissues at various timepoints of interest and staining for biochemical markers, or using mechanical techniques such as atomic force microscopy or

shear rheology to examine tissue stiffness [2], [160], [161]. Additionally, the dimensionality of the model must be considered. 2D models are commonly used as they are easy to use and highly reproducible, but lack complex cell-cell and cell-matrix interactions [2], [147]. 2D cultures are typically also grown on tissue culture plastic, which has a high substrate stiffness and can influence cell phenotype and tissue mechanics [162]. Benefits of 3D models include the facilitation of these cell-cell and cell-matrix interactions, as well as hold greater physiological relevance with increased cellular migration, spatial organization and replication of oxygen, nutrient and growth factor gradients (**Figure 1.8**). There are a number of 3D models, ranging from self-assembled organoids, systems bio-fabricated with scaffolds or on chips, perfusion models, and organ-derived tissue slices [147].

Perhaps the most relevant model type within this context are organoids, which are complex structures of organ-specific cell types. With applicability for toxicology or drug screening and personalized medicine, organoids have better tissue organization than 2D cultures; however, limitations include lack of immune components and other cells, an absence of mechanical cues, and lack of tissue vascularization and perfusion- as such, they are unable to fully recapitulate disease processes [147], [163], [164]. Organoid models have the potential to be improved upon to become more physiologically relevant. For one example, Tan *et al* created a spheroid model mixing different ratios of fibroblasts with human macrophages in order to implement an immune component. They determined the optimal ratio of fibroblasts to macrophages was 16:1, and that macrophages polarized toward a proinflammatory M1 phenotype, subsequently providing greater fibroblast activation and the highest expression of fibrosis-related genes such as collagens I and III, αSMA, and TGF-β1 [165].

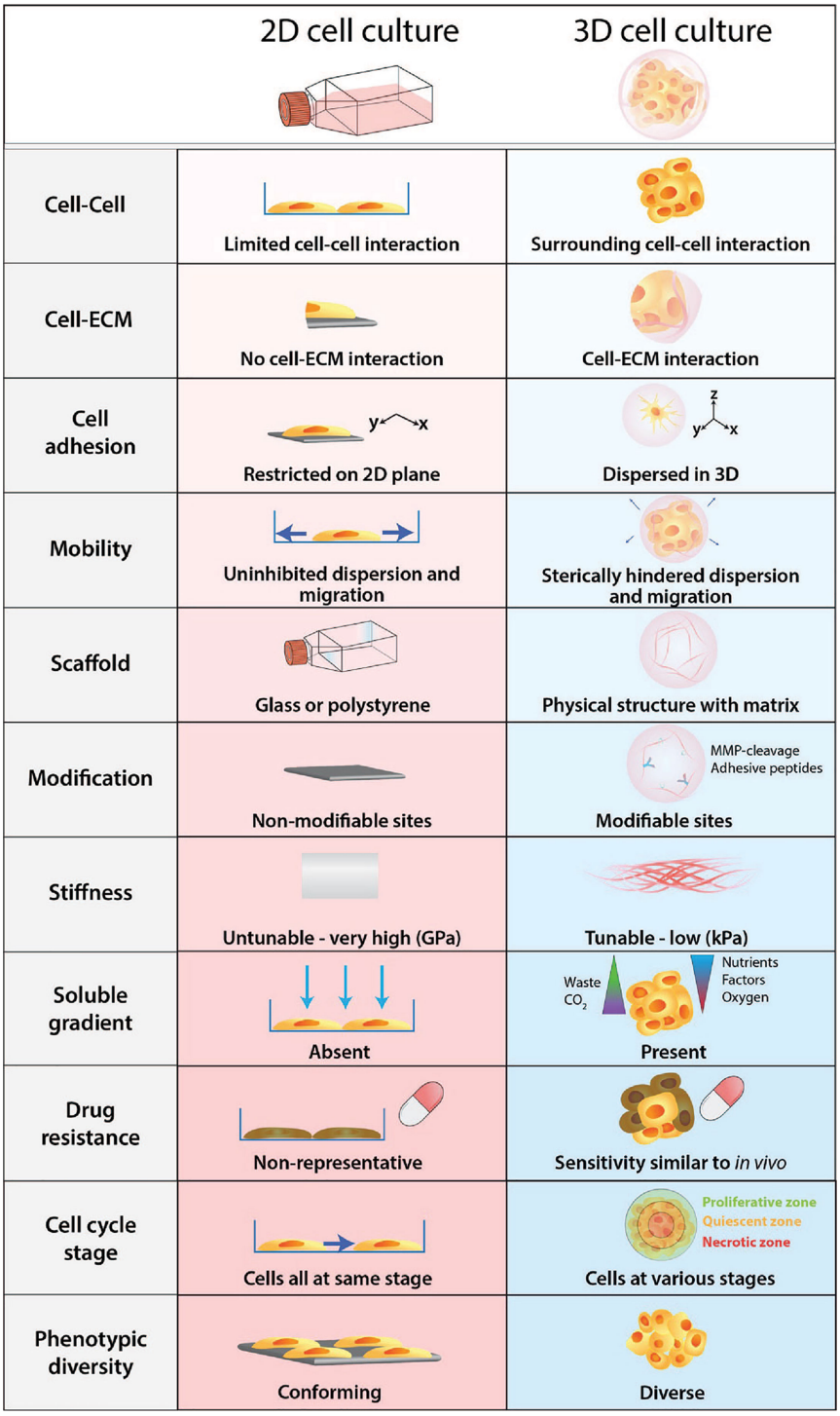


Figure 1.8: Tissue culture properties vary depending on dimension. 2D cell culture is very commonly utilized due to its ease of use, but there are many properties of 3D cell cultures that allow for greater recapitulation of human physiology. Incorporation of as much of these properties as possible will allow for the creation of more predictive models. Adapted from Law et. al [170]

1.10 Specific Aims

Improved models of fibrosis are necessary to both further understanding of the disease etiology as well as develop and evaluate potential therapeutics. As fibrosis is an end result of numerous inflammatory conditions and develops when normal wound healing processes become dysregulated, it is well accepted that there is a complex interplay between the physical matrix and the immune system. Towards that end, we propose that the fibrotic phenotype is a function of the inflammatory environment. We further believe key features of fibrosis can be replicated and induced *in vitro* in a more predictive manner than currently utilized with the incorporation of an immune system component and with consideration taken to the biomechanics of the diseased tissue.

Specific Aim 1- Elucidate how soluble immune components TGF-β1 and IL-13 alter the biomechanical properties of an *in vitro* 3D ring tissue model to induce a fibrotic phenotype.

Fibrosis develops in response to impaired wound healing, a key component of which is involvement of the immune system, where emphasis is on functioning to clear tissue debris as well as signaling for matrix-remodeling effector cells. Thus, a complete, physiologically relevant model of fibrotic diseases must allow for the incorporation of immune system components; this is intrinsic in *in vivo* models, but a concerted effort must be made in development of *in vitro* models. Additionally, when assessing usefulness of a model, it is important to remember that one of the most critical outcomes of the disease is increased tissue stiffness due to uncontrolled deposition of collagen. Thus, the biomechanics of a fibrotic organ should be a phenotypic hallmark for disease progression or regression, as it is more relevant to organ function and

patient prognosis than many other commonly used clinical biomarkers. As a result, it is necessary to incorporate an immune system component in any model, as well as utilize biomechanics capable of describing strength and stiffness to inform of the development of a fibrotic phenotype.

Specific Aim 2- Quantitatively and qualitatively characterize ring tissues treated with inflammatory TGF-β1 and IL-13 in order to understand what changes are occurring temporally, as determined by collagen and DNA content analysis, histology, and second harmonic generation imaging.

Wound healing takes place over the course of 10 days to 12 months depending on the location and severity of the wound as well as the efficiency of the repair process. Therefore, the ability to maintain tissues in culture long term is valuable, in order to track when changes are taking place that would result in development of disease. It is understood that alterations in biomechanics during the development of a fibrotic phenotype are due to some modification of cell or matrix structure or function; thus it is important to determine whether the changes occurring pertain to cell volume or number, the amount of collagen deposited, or the general architecture and organization of the tissue and matrix. To gain that understanding, it is necessary to utilize appropriate biochemical assays and visualization techniques to better characterize the tissue environment.

Specific Aim 3- Determine the molecular mechanisms by which the combination of TGF-β1 and IL-13 facilitates the development of a fibrotic phenotype in a 3D ring tissue fibrosis model, using gene expression analysis.

The functionality of the extracellular matrix, whether in a healthy or diseased state, relies on the complex interplay between cells, structural components and signaling molecules. To better understand the etiology of disease as well as to guide the development of new therapeutics, knowledge of pathways involved in molecular development of a fibrotic phenotype is critical. As an example, if the interaction of the two cytokines result in a synergistic effect, extrapolation of that mechanism may be informative. Additionally, extrapolation of which pathways are upregulated or downregulated in response to profibrotic stimuli may help with narrowing down molecular targets for drug development. Similarly, if a therapeutic candidate proves to be effective in ameliorating disease progression, it would be valuable to understand how in order to prevent unwanted off-target effects. Therefore, it is necessary to examine changes in gene expression levels under a number of conditions through techniques such as PCR in order to tease out the molecular involvement of key players contributing to major phenotypic changes in the fibrotic disease state.

1.11 Tables

Drug	Target, Use, and MOA	Anticipated Effect	Reference(s)
Doxycyclin	Antibiotic that targets collagen; works through TGF-β	Inhibit collagen; decrease mechanics	[171]
Nintedanib	Tyrosine kinase inhibitor which acts on FGFR, VEGFR and PDGFR; used to treat IPF	Decreases collagen; decrease mechanics	[172], [173]
Pirfenidone	Acts as an anti-inflammatory and antifibrotic, affecting collagen synthesis; used to treat IPF	Decrease fibroblast proliferation, possibly decrease mechanics	[9], [10]
Ciprofloxacin	Fluoroquinolone antibiotic that affects elastin, LOX, MMPS	Fragments elastin, decreased LOX, increased MMPs decrease mechanics	[174]
Doxorubicin	Chemotherapeutic that affects collagen; inactivates prolyl-4-hydroxylase in human skin fibroblasts	Inhibits collagen chain assembly; decrease mechanics	[175]
Losartan	Angiotensin II receptor (ATR) blocker	Inhibits fibrosis; inhibits specific MMPS	[4]
Cis-4-hydroxy-l-proline	Proline analogue that affects collagen; enhances procollagen synthesis	Decrease collagen synthesis; decrease mechanics	[176]
Acidic dipeptide hydroxamate	Proline analogue that affects collagen; limits production of functional collagen	Decrease collagen; decrease mechanics	[12], [13]
D-penicillamine	BMP-1 inhibitor; inhibit cleavage of C-terminal peptides preventing collagen fibril formation	Decrease collagen synthesis; decrease mechanics	[175]
Imatinib	Calcium channel blockers; affect collagen by blocking L type calcium channels	Inhibit collagen expression/accumulation; decrease mechanics	[4]
Odanacatib	Recombinant human TGF-β3; affects collagen/fibronectin; reduces deposition of ECM components like collagen/fibronectin and promotes organization of alignment	Decreased fibrosis/better organization; may decrease or modulate mechanics	[4]

Table 1.1: A variety of small molecules and drugs have an effect on the ECM, either directly or indirectly. This list is not meant to be exhaustive nor exclusive, but rather to illustrate the wide variety of molecules that can affect many different facets of the ECM in many different ways.

Organ	Pathways and Processes	Diseases	Drugs	Summary of Effectiveness
Heart	Aldosterone antagonism, TGF-β antagonism, RAS inhibition, cGMP inhibition, inhibition of cholesterol synthesis, inhibition of Na-K-Cl cotransporter	Heart failure, cardiomyopathy, hypertrophic cardiomyopathy, cardiomyopathy induced by type 2 diabetes, heart failure or cardiomyopathy induced by hypertension	Spironolactone, epleronone, canrenone, pirfenidone, sildenafil, statins, ACE inhibitors, ARBs, torsemide, MRAs	ACE inhibitors, ARBs, and MRAs are associated with decreased fibrosis on MRI and decreased arrhythmogenesis (the latter suggests effects of drugs on fibrosis)
Liver	RAS inhibition, inhibition of collagen synthesis, inhibition of effector-cell fibrogenesis, inhibition of oxidative stress, signaling of PPAR γ-agonists	Many diseases of the liver	ACE inhibitors, ARBs, colchicine, interferon γ-1b, vitamin E, pioglitazone, farglitazar	Specific antifibrotic agents listed have generally been ineffective in halting or reversing fibrosis
Kidney	RAS inhibition, aldosterone antagonism, TGF-β antagonism, Nrf2 pathway	Primarily renal diseases related to hypertension or diabetes	ACE inhibitors, ARBs, spironolactone, pirfenidone, bardoxolone	ACE inhibitors and ARBs are moderately effective in slowing progression of diabetic nephropathy (indirectly suggesting effects on fibrosis)
Lung	TGF-β antagonism, direct inhibition of effector-cell fibrogenesis, multikinase inhibition, inhibition of oxidative stress	Primarily idiopathic pulmonary fibrosis	Pirfenidone, interferon γ-1b, bosentan, ambrisentan, macitentan, nintedanib, acetylcysteine	Pirfenidone and nintedanib led to improvements in clinical outcomes
Skin	Endothelin-receptor antagonism, multikinase inhibition	Scleroderma, nephrogenic systemic fibrosis	Bosentan, imatinib mesylate	Small studies show modest effects

Table 1.2: There is a great need for the development of effective antifibrotic drugs. This table illustrates several of the processes by which fibrosis develops, as well as currently available drugs and their efficacy. Development of therapeutics in this space is challenging for several reasons. As seen in the processes for development of the disease, there is significant overlap and conservation of susceptible pathways among different organ systems, which may be useful when considering development of broad-spectrum therapeutic targets. However, attempting to inhibit different cytokines and growth factors can have unintended effects on other cell types and tissues; context is also important, as some cytokines that have profibrotic effects in one scenario may also have antifibrotic and regenerative properties in another. Currently, therapies developed specifically to be antifibrotics have been found to be at most mildly to moderately effective. Adapted from Rockey *et al*. [177]

Murine models	Pros	Cons
Bleomycin	Early molecular signature most similar to accelerated acute phase of IPF in humans	Patchy, young mice resolve spontaneously unless repeatedly doses
Silica	Good model of lung injury in humans and persistence of fibrotic lesions	Lack of reproducibility, difficult delivery, prolonged time to fibrosis, absence of usual interstitial pneumonia (UIP)-like lesions
Asbestosis	Recapitulates asbestos exposure in human lung fibrosis	Inhalation model requires at least a month for fibrosis to develop. Single intratracheal dose leads to central fibrosis rather than subpleural, unevenly distributed between lungs
Cytokine overexpressing	Ability to dissect downstream signaling events relevant to specific fibrotic-inducing cytokines	Models limited to dissecting specific pathways, rather than recapitulating the complexity of human disease
Fluorescent isothiocyanate	Relatively reproducible and persistent fibrotic phenotypes	Lack representative UIP and inflammatory infiltrates preceding fibrosis
Radiation induced	Results in fibrosis, not pneumonitis if B6 mice are used	Need to wait a long time for development of fibrosis
Familial models	Gave insight on telomere and telomerase gene involvement in IPF	May produce a susceptible phenotype, requiring a second hit
Humanized (NOD/SCID mice)	Can afford insight into role of different fibroblast populations, dissects the contribution of epithelial-fibroblast crosstalk in the absence of immune cells	May not be representative of human disease where immune cells play a role. Expensive and requires specialized housing
Domestic animals	**Pros**	**Cons**
Dogs	Usually present in middle to old age. IPF in Westies shares some features of human disease; foci with severe lesions, histological criteria more typical for UIP may be present. Spontaneously develop ILD	The diffuse interstitial lesion, present in all affected Westies, histologically resembles fibrotic NSIP in man
Cats	Anatomy of distal lung similar to humans. UIP-like disease. Spontaneously develop ILD	Strain-dependent
Donkeys	Spontaneously develop ILD	Majority of cases of APF share key pathological features with human pleuroparenchymal fibroelastosis not IPF
Horses	Spontaneously develop ILD. Overlapping features of pulmonary fibrosis including weight loss and characteristic radiologic findings	Pathology not the same as IPF

Table 1.3: While animal models are commonly used to study diseases and test the safety and efficacy of potential therapeutics, there are simultaneously advantages and disadvantages. Some methods of inducing injury to stimulate the development fibrosis, such as treatment with bleomycin, are effective in creating a physiologically relevant model. Others, however, lack representative characteristics of disease in humans, do not develop fibrosis in a reproducible manner, or are not realistic in timeline to disease manifestation. Overall, in addition to ethical issues involved in the use of animals, the resulting studies may be expensive and unreliable. These model characteristics should be considered when determining an approach for study of a pathway or drug. Adapted from Tashiro *et al*. [138]

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Chapter 2

TGF-β1 requires IL-13 to sustain collagen accumulation and increasing tissue strength and stiffness

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2.1 Abstract

Fibrosis is a multifactorial process characterized by the excessive accumulation of extracellular matrix (ECM), increased tissue stiffness, and decreased elasticity. To understand how growth factor mediated dysregulation of fibroblasts leads to alterations in the biomechanics of the ECM, we've developed a new long term model whereby human fibroblasts form a fibrous 3D ring-shaped tissue whose tensile strength and stiffness steadily increases over three weeks. As the rings compact, cellularity and total DNA decrease, whereas total collagen accumulates. TGF-β1 stimulates collagen accumulation and increases ring biomechanics at day 7, but these increases stall and decline by day 21. When treated with IL-13, a cytokine exclusive to the immune system, there are no significant differences from control. However, when TGF-β1 is combined with IL-13, collagen levels and ring biomechanics increase over the three weeks in culture to levels higher than TGF-β1 alone. Gene expression is differentially regulated by growth factor treatment over the duration in culture and suggests that increased collagen accumulation is not due to upregulation of collagen gene expression. These results suggest that TGF-β1 requires a second signal, such as IL-13, to sustain the long-term pathological increases in collagen accumulation and biomechanics that can compromise the function of fibrotic tissues.

2.2 Introduction

Fibrosis and excessive accumulation of extracellular matrix (ECM) is a characteristic of chronic inflammatory diseases, affects most organ systems and accounts for nearly 30% of deaths in the developed world [1]–[4]. Central to fibrosis is the fibroblast, the cell type responsible for the excessive production of the collagenous ECM that compromises organ

function, often by altering its biomechanics [2], [5]–[8]. Dysregulation of the fibroblast due to signals emanating from injury and inflammation are drivers of fibrosis. However, the phases of wound healing and the chronic inflammation that precedes fibrosis occurs over weeks and is a complex interplay between fibroblasts and multiple cell types of the immune system [9], [10]. Growth factors and cytokines acting as autocrine and paracrine signals is one means by which the fibroblast undergoes activation to a pathological state [6],[11].

Transforming growth factor beta-1 (TGF-β1) is a complex multi-functional growth factor central to the development of fibrosis [12], [13]. Synthesized and secreted by a wide variety of cell types including fibroblasts as well as various immune cells, TGF-β1 is produced in a latent form sequestered in the ECM [14], [15]. Release and activation of TGF-β1 occurs via by multiple pathways [14]. Once activated, TGF-β1 binds its receptor and signals through the canonical ALK5/SMAD3 pathway [16]. Actions of TGF-β1 linked to fibrosis include stimulation of fibroblast migration and proliferation, activation of fibroblasts to myofibroblasts, as well as the stimulation of collagen production [12], [17], [18].

Interleukin -13 (IL-13) is a pleotropic cytokine that has also been implicated in fibrosis, but unlike TGF-β, IL-13 is not produced by fibroblasts [6], [16]. IL-13 expression is confined exclusively to cells of the immune system including T helper type 2 cells, natural killer T cells, macrophages, basophils, eosinophils and mast cells [19]. IL-13 signals the JAK/STAT6 pathway by binding to a Type II receptor consisting of IL-13Rα1 complexed with IL-4Rα [20]. Interestingly, another protein, IL-13Rα2, binds IL-13 with even higher affinity and is thought to act as a decoy receptor [21]–[23]. IL-13 is capable of activating fibroblasts, stimulating proliferation, and increasing collagen production [24].

In this paper, we used a new *in vitro* model of the human ECM to investigate the effects of TGF-β1 and IL-13. When seeded into circular agarose molds, human fibroblasts self-assemble and form a three dimensional (3D) ring-shaped tissue with a circumferentially aligned collagen-rich ECM with measurable biomechanics (ultimate tensile strength or UTS, and maximum tangent modulus or MTM) that increases steadily over a three week period [25], [26]. TGF-β1 stimulates collagen production and increases ring biomechanics at week one, but these increases stall and decline by week three. IL-13 alone has no significant effect, but when combined with TGF-β1, collagen levels and ring biomechanics increase over the entire three weeks to levels higher than TGF-β1 alone. These results suggest that TGF-β1 requires a second signal such as IL-13 to sustain increases in collagen and biomechanics.

2.3 Materials & Methods

2.3.1 Cell source and culture conditions

Fibroblast tissue rings were comprised of juvenile normal human dermal fibroblasts (jNHDF) obtained commercially (PromoCell, Heidelberg, Germany). Cells were cultured in two dimensions (2D) in Dulbecco's Modified Eagle's medium (DMEM) with high glucose, phenol red, L-glutamine, and sodium pyruvate (#11995065, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (#100-500, Gemini Bio, West Sacramento, CA) and 1% penicillin/streptomycin (#091670249, Thomas Scientific, Swedesboro, NJ) at 10% CO_2 and 37 °C. Cells were propagated (passages 4-8) utilizing a routine trypsin protocol. Cells were briefly rinsed with phosphate buffered saline (PBS)(#SH30256.FS, Thomas Scientific) after which 0.05% trypsin (# SH3004201, Thermo Fisher Scientific) in PBS was added for 5 minutes

to liberate the cells. Medium containing FBS was used to quench the trypsin. The cell suspension was collected, centrifuged (220g for 5 minutes), resuspended in fresh medium, counted, and seeded. Cells were propagated in T-175 flasks (#10-126-13, Fisher Scientific, Hampton, NH) at a density of $0.5x10^6$ cells/flask.

2.3.2 Formation of ring tissues

Molds and molded agarose with a circular trough (5 mm diameter) for the formation of ring tissues have been previously described [26]. Briefly, computer aided design (CAD)(SolidWorks, Concord, MA) and rapid prototyping (Protolabs, Maple Plain, MN) were used to design and fabricate stainless steel molds each with a circular feature and an accompanying aluminum base that positioned each mold into a single well of a standard 24-well plate.

Each well was filled with 1.5 mL of molten, sterile agarose (2% w/v)(#BP160-500, Fisher Scientific, Hampton, NH) in PBS. A stainless steel mold was inserted into each well and the agarose allowed to cool and gel for 15 minutes. Upon removal of the molds, each well contained molded agarose with a 0.75 mm wide cylindrical trough surrounding an agarose peg (5 mm diameter). Prior to the addition of cells, gels were equilibrated with serum-free DMEM with 1% penicillin/streptomycin through a series of medium exchanges at least 24 hours prior to use. For ring tissue formation, an optimized culture medium (50:50) was used as previously described [26]. This 50:50 medium contained an equal amount of high glucose DMEM (#11995065) supplemented with 0.1 mM 2-phospho-l-ascorbic acid trisodium salt (#49752, Sigma- Aldrich, St. Louis, MO), 50.0 μg/mL L-proline (#BP392-100, Fisher Scientific), and 1% penicillin/streptomycin and an equal amount of advanced DMEM (#12491015, Thermo Fisher)

supplemented with 4 mM GlutaMax (#35-050-061, Fisher Scientific) and 1% penicillin/streptomycin. To form ring tissues, each agarose mold was seeded with $3x10^5$ cells/ring. Medium was changed every 2-3 days.

Prior to adding TGF-β1, IL-13 or TGF-β1 plus IL-13, rings were allowed to self-assemble for a period of 24 hours. TGF-β1 and IL-13 from Peprotech (#100-21 and #200-13, respectively) were resuspended and aliquoted according to manufacturer's protocols. Briefly, TGF-β1 was reconstituted in 10 mM citric acid, pH 3.0 (Cell Signaling Technologies, #9871L) and diluted in 0.1% (w/v) final concentration of bovine serum albumin (BSA) in PBS. IL-13 was reconstituted in 0.1% (w/v) concentration of BSA in PBS. (#A3294, Sigma-Aldrich). Each well has 1.5 mL of an agarose hydrogel equilibrated with culture medium and 1 mL of removable culture medium. Growth factors were added to 50:50 medium to achieve a final concentration of 10 ng/mL after equilibration with the agarose gel. Medium was refreshed 3x/weekly.

2.3.3 Mechanical testing

The biomechanics of rings were tested as previously described [26]. Briefly, conventional (*x, y*) and side view (*z*) images of the rings on the agarose peg were obtained using a Nikon Eclipse Ts2 microscope (Nikon, Tokyo, Japan). Measurements of ring thickness (*x, y, z*) were used to calculate cross-sectional area (CSA) to normalize mechanical values. Rings were dissected from their agarose molds and mounted on custom grippers fitted to an Instron 5943 (Norwood, MA) equipped with a 5N (5mN resolution) load cell. This custom gripper has two semi-circular pegs that create a circular diameter of 3 mm and is fabricated from glass-filled nylon (#PA614-GS, Protolabs). Rings mounted on this gripper were submerged in a PBS bath at 37°C. Grippers were

brought to an initial starting distance of 5 mm, and test protocols were run such that the two pegs moved away from each other, displacing the ring at a rate of 0.1% initial length per second. Load (N) and extension (mm) were sampled at 20 Hz, and tests were terminated when the rings broke, which corresponded with a detected 40% drop in load. Broken rings were snap frozen in liquid nitrogen and stored at -80°C for subsequent collagen and DNA measurements.

Data from the tensile tests were analyzed as previously described [26] using a custom Python 3.6 code run on a Jupyter Notebook. The mechanical testing protocol was adapted from Adebayo, *et al*. and Gwyther, *et al*., while the means of analysis was adapted from Ristaniemi *et al* [46]–[48]. Essentially, raw data was filtered to remove the drag force generated by the grippers without a loaded sample. The tissue was considered to be in tension when the raw load was greater than the resolution of the load cell (5mN). The maximum tangent modulus (MTM) and ultimate tensile strength (UTS) were derived from examination of the engineering stress ($N/2A_0$) and engineering strain ($\Delta L/L_{\text{gauge}}$), where A_0 is the starting cross-sectional area. Three to four technical replicates from three independent biological experiments were run for each condition for an *n* equal to ten.

2.3.4 Histology

Rings in their agarose mold were fixed in 10% buffered formalin and kept at 4°C until embedding. To prepare for embedding, rings on their pegs were dissected away from the gel and the agarose peg with ring was placed upright in a 24-well plate. Fresh 2% w/v agarose/saline solution cooled to just above the gelling temperature was added to each well to encapsulate the agarose peg with ring. After gelling, the agarose puck with encapsulated ring was transferred to a cassette and paraffin embedded. Embedded samples were cut into 5-8 μm sections using a Leica

RM2265 microtome (Leica Microsystems, Wetzlar, Germany). Slides were stained with hematoxylin and eosin (Richard-Allan Scientific; Thermo Scientific) or Masson's Trichrome (Electron Microscopy Sciences, Hatfield, PA) according to the manufacturer's protocols. All slides were viewed and digitized using an Olympus VS200 Slide Scanner (Olympus, Tokyo, Japan) with associated VS200 ASW software followed by viewing in OlyVIA (Olympus, open access).

2.3.5 Multiphoton second-harmonic generation microscopy

An Olympus FV-1000-MPE multiphoton microscope (Olympus) equipped with a Mai Tai HP tunable laser set to excitation wavelength at 790 nm and fitted with a 405/40 filter cube was used to visualize fibrillar collagen via a second-harmonic signal. Tissue rings were fixed with 10% formalin in their agarose gels and kept at 4°C prior to imagining. Rings were washed with PBS, moved to a petri dish (60 mm), submerged in PBS and imaged in their agarose molds using a 25x dipping objective (numerical aperture 1.05, working distance of 2 mm). Images were obtained using FV10-ASW software (Olympus) and later read using OlyVIA.

2.3.6 Quantification of collagen and DNA

To quantify total collagen, rings were solubilized and levels of hydroxyproline measured using a colorimetric assay [49]. Briefly, individual rings were digested in 125 ug/mL papain (#P4762, Sigma Aldrich) in water at pH 6.5 at 65°C for 10 days. Samples were treated with a 1:1 volume of 4M NaOH (#S318-1, Fisher), incubated at 120°C for an hour and neutralized with an equal volume of 4M HCl (#SA56-1, Fisher). Chloramine T solution (#S318-1, Fisher) was added and

samples incubated at room temperature for 20 minutes. Ehrlich's reagent (#LC140802, LabChem) was added and samples incubated at 65°C in water for 30 minutes. Samples were transferred to a 96-well plate and absorbance at 550 nm read using a UV plate reader (SpectaMax Plus 384, Molecular Devices, San Jose, CA). Values of collagen (ug/ ring) were derived from a standard curve of type I rat tail collagen (#5056, Advanced Biomatrix, Carlsbad, CA).

Total DNA content was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (#P11496, Invitrogen, Waltham, MA) per the manufacturer's protocol using the papain digested samples prepared for collagen analysis. Briefly, samples were incubated with the picogreen solution at room temperature for 5 minutes, transferred to a 96-well plate and fluorescence (480 nm excitation, 520 nm emission) measured using a fluorescent plate reader (SpectraMax Gemini XS, Molecular Devices). Values of DNA (ng/mL) were derived from a standard curve of λ DNA ((#P11496, Invitrogen). For each assay, three to four technical replicates from three independent biological experiments were run for each condition for an *n* equal to ten.

2.3.7 qPCR

Rings (3 per tube) for qPCR were snap frozen in liquid nitrogen and stored in -80°C until use. Unless otherwise specified, all reagents were obtained from a Quick-RNA Microprep Kit (#R1051, Zymo Research, Irvine, CA) and all consumables were DNase- and RNase-free. To extract RNA, rings on dry ice were mechanically disrupted using a motorized pellet pestle homogenizer (#12-141-361, Fisher) and associated tubes and pestles (#K749520-0090, Fisher). Rings were homogenized and placed on dry ice, allowed to refreeze, and homogenized again for

a total of 3-5 cycles. DNA/RNA shield was added prior to the last manual homogenization. Proteinase K solution (#D3001-2-20, Zymo) was added and samples incubated at room temperature for 2 hours. RNA lysis buffer (1:1) was added and samples incubated at room temperature for 10 minutes. Samples were centrifuged to remove particulate debris, moved to new tubes, and RNA extraction was performed according to manufacturer's protocols. Eluted total RNA was aliquoted and stored at -80°C until quantified (NanoDrop2000C, Thermo Fisher).

cDNA was synthesized using the Thermo Vero cDNA Synthesis Kit (#AB1453B, Thermo Scientific) according to manufacturer's protocols. Briefly, 50 ng of RNA per sample were added to strip tubes and heated to 70°C for 5 minutes to disrupt the RNA secondary structure. A master mix cocktail containing cDNA synthesis buffer, dNTP mix, random hexamers, RT enhancer, and Verso enzyme mix was added and samples incubated at room temperature for 10 minutes to start the annealing process. Samples were loaded onto a Bio-Rad CFX96 qPCR machine (Bio-Rad, Herculues, CA) and incubated at 42°C for an hour to facilitate synthesis, followed by 2 minutes at 95°C to deactivate the enhancer. Samples were quantified (NanoDrop) and stored at -80°C.

cDNA was diluted to a working stock of 50 ng/ul. All primers were ordered as custom oligos, 25 nmol, no modifications, desalted, and dry formulation (Thermo Fisher). All primers were reconstituted and diluted as specified by the manufacturer to the final concentration of 4 uM. For each reaction, a cocktail of Yellow Sample Buffer and SYBR Green from PowerTrack SYBR Green Master Mix (#A46110, Thermo Scientific) was used. 100 ng of cDNA was added, along with forward and reverse primers, and the final reaction mixture was brought to volume with nuclease-free water. Each reaction was pipetted to a white-walled 96-well plate in either

duplicate (housekeeping) or triplicate (gene of interest). The plate was loaded onto the Bio-Rad CFX96 qPCR machine and a protocol run with 40 alternating cycles of denaturing at 95°C and annealing at 60°C. Data was analyzed using the ΔΔCT method as described in Schmittgen et al [50] and normalized to day 7 control tissues. For all PCR experiments, two technical replicates (containing 3 rings a piece) from three biological experiments were run for each condition for an *n* equal to six.

2.3.8 Statistical analysis

All data were graphically and statistically analyzed using JMP Pro 16 (JMP, Cary, NC). All graphed data is represented as the mean +/- the standard deviation. The Levene's test and the Shapiro-Wilk test were used to test the variance and normality of the data, respectively. When examining statistical significance between multiple groups, a non-parametric Kruskal-Wallis test with post-hoc Dunn's each pair was used. Threshold for significance was set at $p<0.05$ for all tests.

2.4 Results

2.4.1 Human fibroblasts form tissue rings and compact over time.

To form 3D ring tissues, we seeded human fibroblasts into circular agarose troughs (5mm diameter) molded in a 24 well plate as previously described [26]. Unable to attach to the agarose, the cells rapidly aggregated and self-assembled a ring tissue (one per well) within 24 hours. Twenty-four hours after seeding, rings were treated with TGF-β1, IL-13 or TGF-β1 plus IL-13 with untreated rings as a control. The cross sectional area (CSA) of the rings was calculated from measurements of ring thickness from conventional brightfield images (*x, y*) and side view images (*z*) at days 7, 14, and 21 (**Figure 2.1**). At day 7 and 14, the CSA of rings treated with TGF-β1 or TGF-β1 plus IL-13 were increased compared to control rings or rings treated with IL-13 alone. By day 21, the CSA of control, TGF-β1, and IL-13 treated rings were comparable and only rings treated with TGF-β1 plus IL-13 were increased. Over the course of three weeks, the CSA of control, TGF-β1, and IL-13 treated rings declined in size suggesting tissue compaction, whereas the CSA of rings treated TGF-β1 plus IL-13 were unchanged.

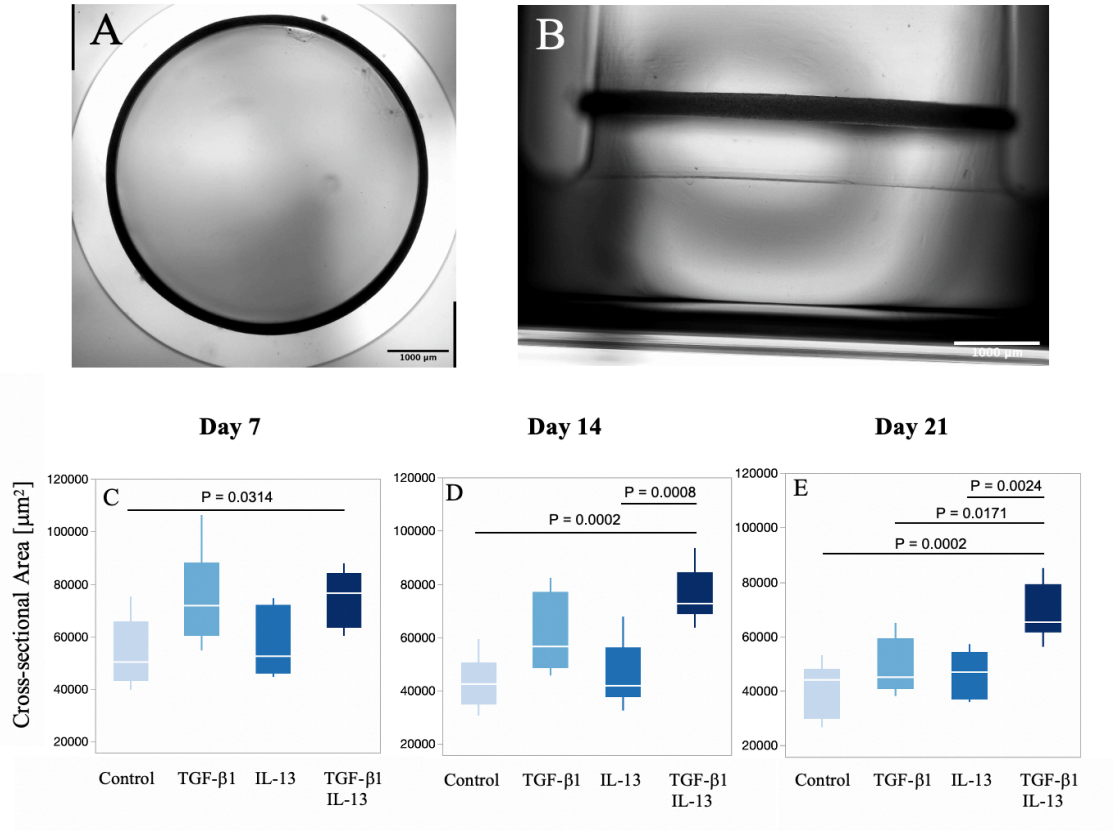

Figure 2.1: TGF-β1 plus IL-13 sustains an increase in the cross sectional area of ring tissues**.** Human dermal fibroblasts ($3x10^5$/ring) were seeded in circular troughs (5mm inner diameter) molded in agarose that had been equilibrated with cell culture medium. Control rings and rings treated with TGF-β1, IL-13 or TGF-β1 plus IL-13 were imaged at days 7, 14, and 21. Ring thickness was measured from conventional images (*x, y*)(A) and side view images (*z*)(B) and used to calculate the cross sectional area (CSA) of the rings. CSA of control rings and rings treated with TGF-β1 or IL-13 decreased over time, whereas rings treated with TGF-β1 plus IL-13 maintained the day 7 increase in CSA (C-E). Scale bars = 1000 µm (Kruskal-Wallis with post-hoc Dunn's test, $p<0.05$. n = 10).

2.4.2 TGF-β1 requires IL-13 to sustain an increase in total collagen.

To quantify total collagen, we measured the levels of hydroxyproline (**Figure 2.2**). Total collagen of control and IL-13 treated rings were comparable and increased slowly over the three weeks with the largest increase occurring over the first seven days. In contrast, the total collagen of rings treated with TGF-β1 or TGF-β1 plus IL-13 increased more quickly. At day 7, both were higher than control or IL-13. However, collagen levels of TGF-β1 rings plateaued at day 14 and declined slightly by day 21, and there was no significant difference compared to control or IL-13. However, when TGF-β1 was combined with IL-13, total collagen continued to increase over 3 weeks and was greater than both tissues from all other conditions at day 21. Similar trends can be seen when collagen density (content as a function of CSA) is examined (**Suppl. Figure 2.8**).

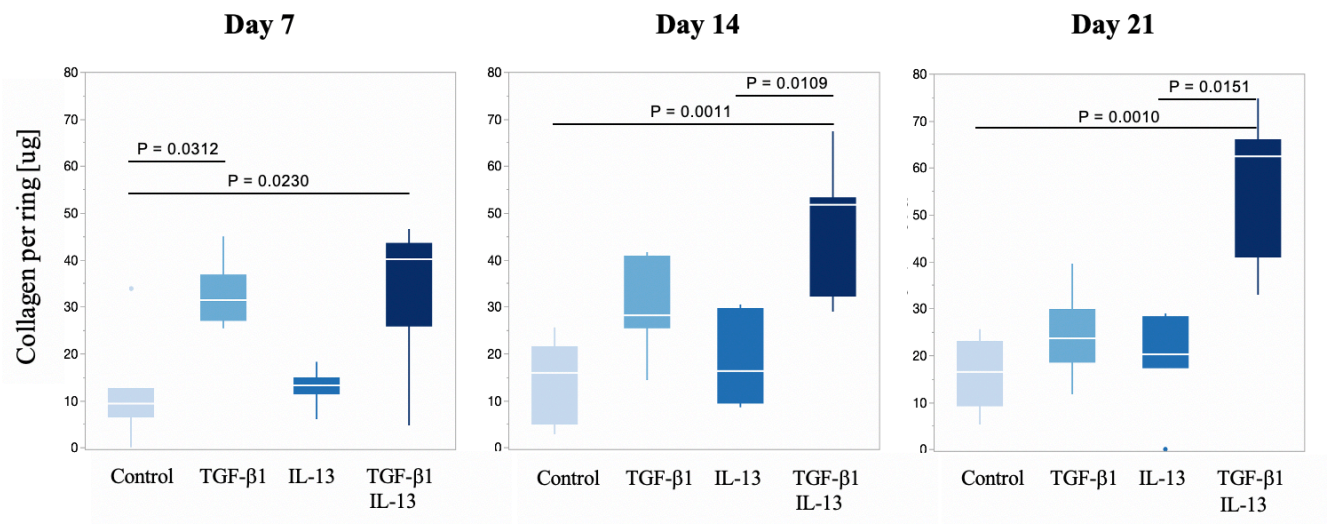

Figure 2.2: TGF-β1 plus IL-13 sustains an increase in the total collagen of ring tissues**.** Control rings and rings treated with TGF-β1, IL-13 or TGF-β1 plus IL-13 were harvested at days 7, 14, and 21 and used to measure total collagen. At day 7, collagen levels of rings treated with TGF-β1 or TGF-β1 plus IL-13 were substantially higher than control rings and IL-13 rings. Although collagen levels increased for control and IL-13 rings at days 14 and 21, rings treated with TGF-β1 plus IL-13 continued to be higher in total collagen versus the other conditions. (Kruskal-Wallis with post-hoc Dunn's test, $p<0.05$. n = 10).

2.4.3 TGF-β1 requires IL-13 to sustain an increase in biomechanics.

To determine if biomechanics was altered by time and treatment, rings were subjected to tensile testing and the resultant stress-strain curves were used to calculate the two metrics that will hereby be referred to as biomechanics: ultimate tensile strength (UTS) and the maximum tangent modulus (MTM)(stiffness)(**Figure 2.3**). All values were normalized to the CSA of each ring. The UTS and MTM of control and IL-13-treated rings increased steadily over the three weeks with IL-13 rings slightly stronger and stiffer by day 21. In contrast, the UTS and MTM of rings treated with TGF-β1 or TGF-β1 plus IL-13 increased quickly. At day 7, both were considerably stronger and stiffer than control or IL-13. But, by day 14, the UTS and MTM of TGF-β1 rings were no different than control or IL-13 and by day 21, TGF-β1 rings were substantially less than control or IL-13. However, when rings were treated with TGF-β1 plus IL-13, UTS and MTM continued to increase over 3 weeks and was greater than all other conditions. Interestingly, these results mirror the time and treatment dependent changes in collagen levels.

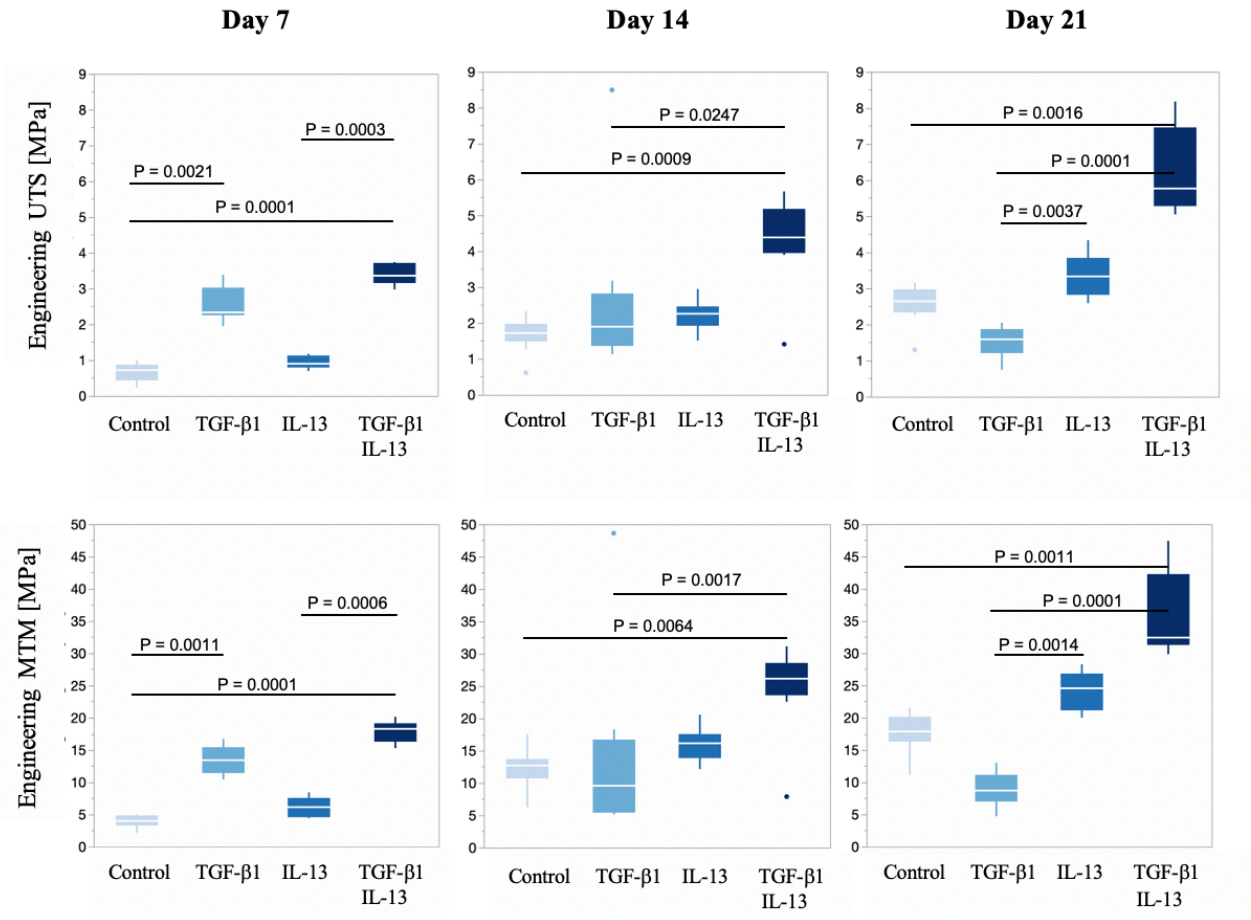

Figure 2.3: TGF-β1 plus IL-13 sustains an increase in the strength and stiffness of rings. Control rings and rings treated with TGF-β1, IL-13 or IL-13 plus TGF-β1 were cultured for 7, 14, and 21 days, released from their agarose molds, mounted on a custom Instron gripper, and subjected to tensile testing to measure ultimate tensile strength (UTS)(strength) and maximum tangent modulus (MTM)(stiffness). The strength and stiffness of control and IL-13 rings increased steadily over the time course, with IL-13 rings being slightly stronger and stiffer by day 21. The mechanics of TGF-β1-treated rings increased quickly and were stronger and stiffer than control or IL-13 at day 7, but their mechanical properties plateaued at day 14, followed by decline at day 21. In contrast, the mechanics of rings treated with TGF-β1 plus IL-13 also increased quickly and were considerably stronger and stiffer than controls at day 7, but continued to gain strength and stiffness for the duration of culture to levels well beyond TGF-β1 alone. (Kruskal-Wallis with post-hoc Dunn's test, $p<0.05$. n = 10).

2.4.4 Cellularity decreases and TGF-β1 increases pyknotic nuclei.

To examine histology, control and treated rings at days 7, 14, and 21 were fixed and paraffin sections stained with hematoxylin and eosin (H&E) or Masson's trichrome (**Figure 2.4**). At day 7, control and treated rings were all highly cellular with substantial amounts of collagen consistent with most collagen synthesized within the first week. Over the course of three weeks, cellularity decreased and the rings became more organized as shown by increased elongation and alignment of cells and the collagen matrix. At day 21, the collagenous matrix of control and IL-13 rings was thinner and more closely spaced than rings treated with TGF-β1 or TGF-β1 plus IL-13.

Interestingly, pyknotic nuclei were evident in control and treated rings at all times points suggesting that the cells were undergoing a process of terminal differentiation (**Suppl. Figure 2.9**). When compared to control and IL-13 rings, the proportion of pyknotic nuclei in rings treated with TGF-β1 or TGF-β1 plus IL-13 appeared to be increased, suggesting that TGF-β1 could potentially be driving this terminal differentiation.

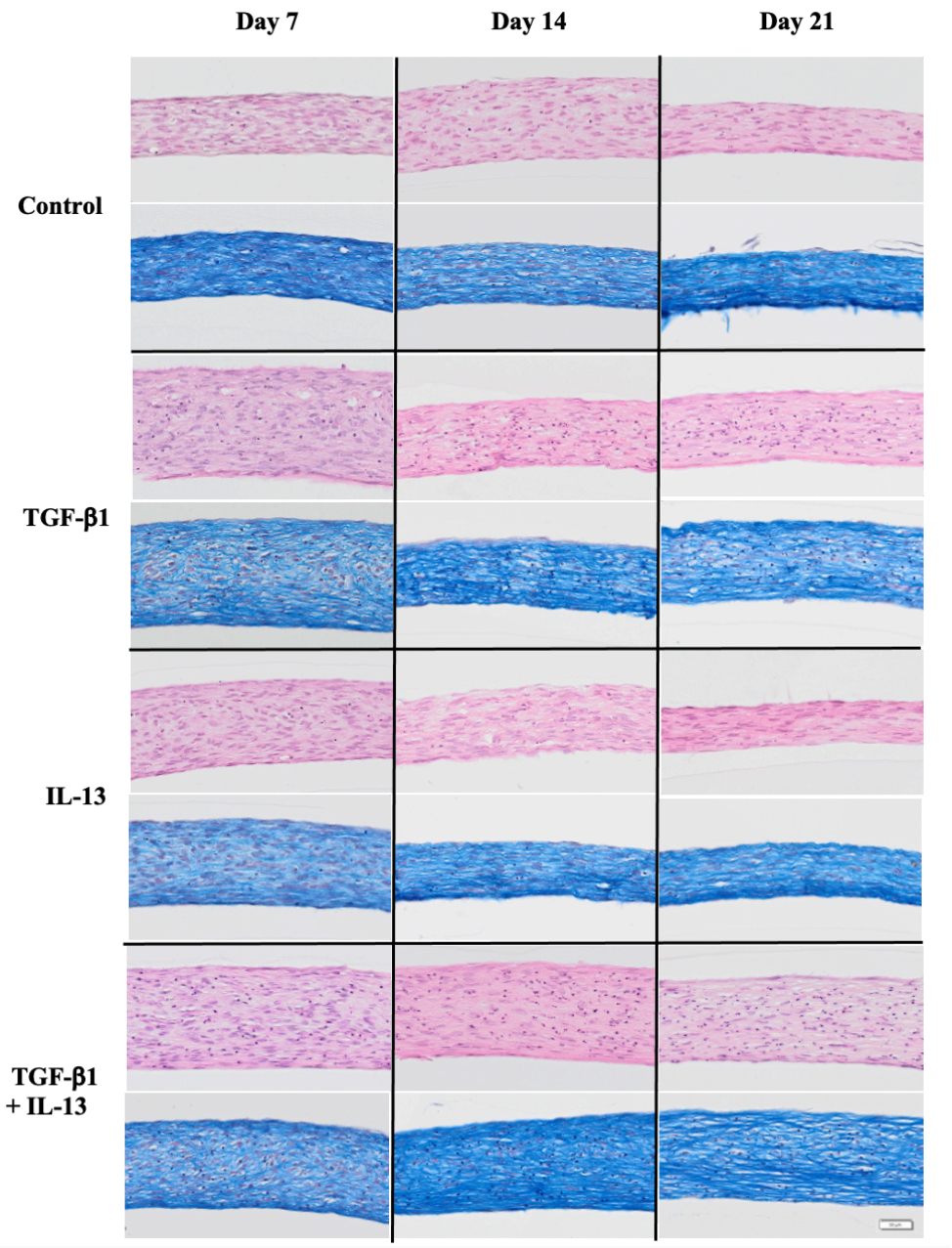

Figure 2.4: Ring tissues deposit collagen, condense over time, and TGF-β1 increases the number of pyknotic nuclei. Control and treated rings were harvested, fixed, paraffin embedded and sections stained with hematoxylin and eosin (H&E)(pink) or Masson's trichrome (MT)(blue). The bottom side of each section is the surface of the ring that contacts the agarose peg. Cell elongation, alignment and collagen deposition are evident as early as day 7 in all conditions and increases with time in culture. Also evident is a decrease in cellularity and an increase in collagen fibril accumulation as the tissues mature from 7 to 21 days. While pyknotic nuclei are present in all tissues, particularly at later time points, the number of pyknotic nuclei appeared increased in rings treated with TGF-β1 alone or TGF-β1 plus IL-13. Pyknotic nuclei are adjacent to healthy elongated nuclei in the interior of the rings suggesting that their formation is TGF-β1 driven and not due to the diffusion limitations of the tissue. Scale bar = 50 μm.

2.4.5 Tissue DNA content decreases, but TGF-β1 increases DNA.

To understand the decrease in cellularity, we measured total DNA of the rings (**Suppl. Figure 2.7**). Total DNA of all conditions decreased slowly over the three weeks and levels of control and IL-13 rings were comparable. However, as early as day 7 and continuing to day 21, total DNA of rings treated with TGF-β1 or TGF-β1 plus IL-13 were considerably higher than control or IL-13. This result correlates with the increased CSA of these rings at day 7. The DNA results are consistent with an overall decline in cellularity, with the caveat that the assay measures total DNA and is unable to distinguish between normal and pyknotic nuclei.

2.4.6 Fibrillar collagen architecture varies with time and treatment.

To examine the organization of the collagen fibers, we used multiphoton second-harmonic generation (SHG) to image the rings (**Figure 2.5**). Isometric *z*-stacks were obtained and representative images presented. Organization of the collagen fibers of control and treated rings increased over time. Interestingly, the fibers of rings treated with TGF-β1 appeared to be thinner and more aligned than control rings. In contrast, the fibers of rings treated with IL-13 or TGF-β1 plus IL-13 were visually more intertwined and mesh-like, especially at day 14.

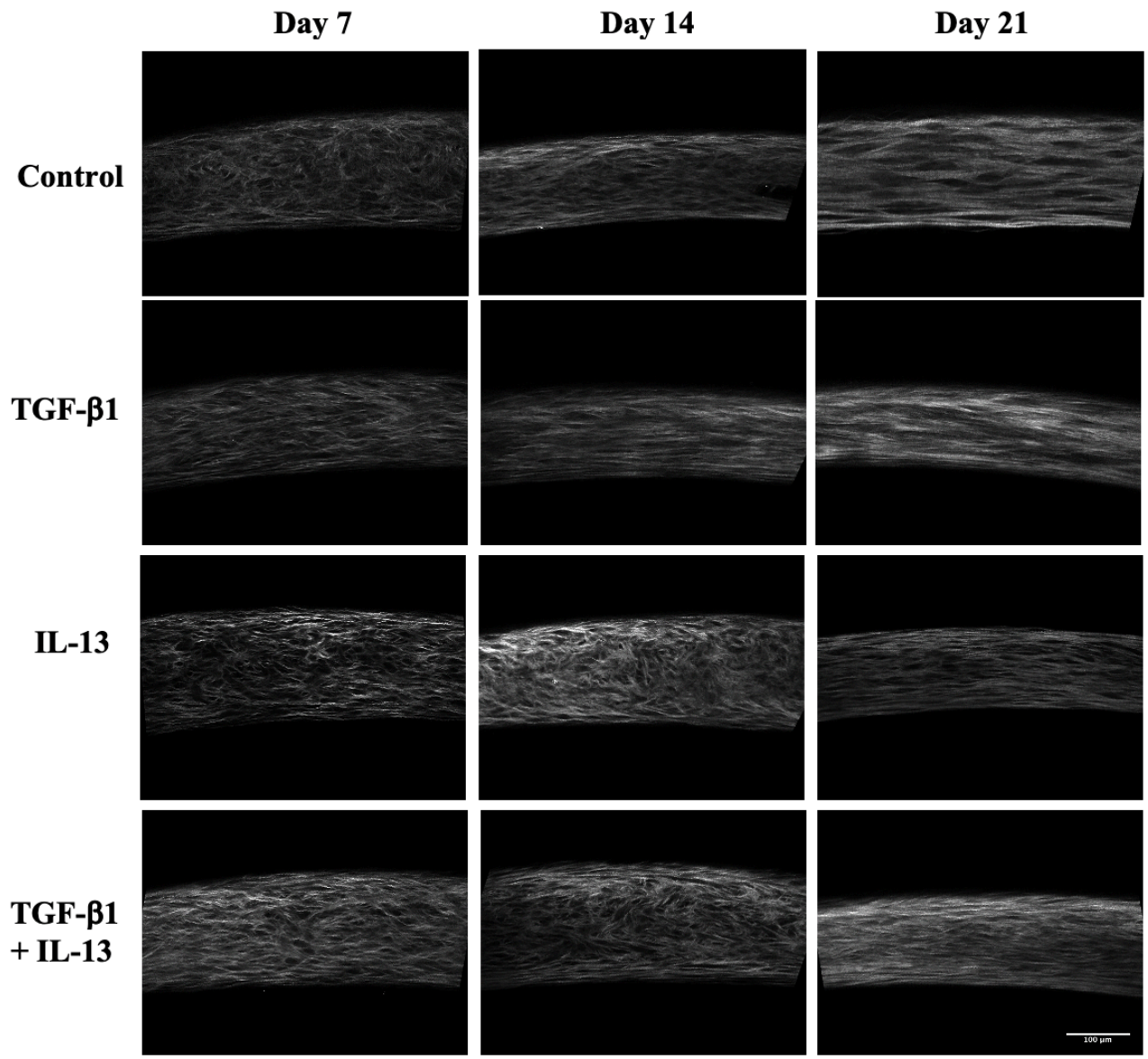

Figure 2.5: The architecture of fibrillar collagen varies with time and treatment. Fibrillar collagen of control and treated rings were imaged using multiphoton second-harmonic generation (SHG) microscopy. Rings were fixed in formalin, submerged in PBS, and imaged *in situ* within their agarose molds. The bottom side of each image is the surface of the ring tissue that contacts the agarose peg. In all conditions, collagen fibrils are evident as early as day 7 and subsequently increase their alignment and maturation with time in culture. Rings treated with TGF-β1 presented fibers that appeared thinner and straighter than control rings. Separately, rings treated with IL-13 as well as TGF-β1 plus IL-13 presented fibers that were more intertwined, creating a mesh-like pattern that was especially evident at day 14. Scale bar = 100 μm.

2.4.7 Gene expression is differentially regulated by TGF-β1 and IL-13.

To determine if rings were actively responding to TGF-β1 and IL-13 over the entire three weeks and to assess if differential gene expression might explain our observations, we performed RT-qPCR on selected genes (**Figure 2.6, Suppl. Table 2.1 & 2.2**). All data was normalized to day 7 control tissues. At day 21, αSMA, a well-known marker of the myofibroblast phenotype, was induced 9.6 fold in TGF-β1 rings and 4.4 fold in rings treated with TGF-β1 plus IL-13, demonstrating that the rings were responsive to TGF-β1. IL-13 alone had no effect on αSMA. Likewise, IL13Rα1, the signaling receptor for IL-13 was induced 2.2 fold by TGF-β1 and IL-13 had no effect, a result consistent with their known actions. Interestingly, the TGF-β1 mediated induction of IL13Rα1 was suppressed when TGF-β1 was combined with IL-13. At day 21, IL13Rα2, the decoy receptor for IL-13, was induced 24.6 fold by IL-13, clear evidence that the rings are responding to IL-13. TGF-β1 alone had no effect on IL13Rα2. Interestingly, TGF-β1 plus IL-13 suppressed this large induction to slightly above control levels (1.5 fold).

2.4.8 Increased collagen is not due to upregulation of collagen gene expression.

In contrast, there were little if any changes to COL1A1 or COL3A1 at any of the time points measured including day 7. Interestingly at day 21, COL6A1 was slightly reduced in control (0.7 fold) and by TGF-β1 alone (0.7 fold), but was slightly induced by IL-13 alone (1.4 fold) and TGF-β1 plus IL-13 (1.6 fold). There were no changes to MMP2 and a slight downregulation of TIMP1 in TGF-β1 treated rings (0.2 fold). Levels of MMP9 were undetectable. TIMP 1 was slightly suppressed by TGF-β1 (0.2 fold) and by TGF-β1 plus IL-13 (0.3 fold), whereas TIMP3 was induced by TGF-β1 alone (4.0- 2.6 fold from day 7 to day 21) and by TGF-β1 plus IL-13

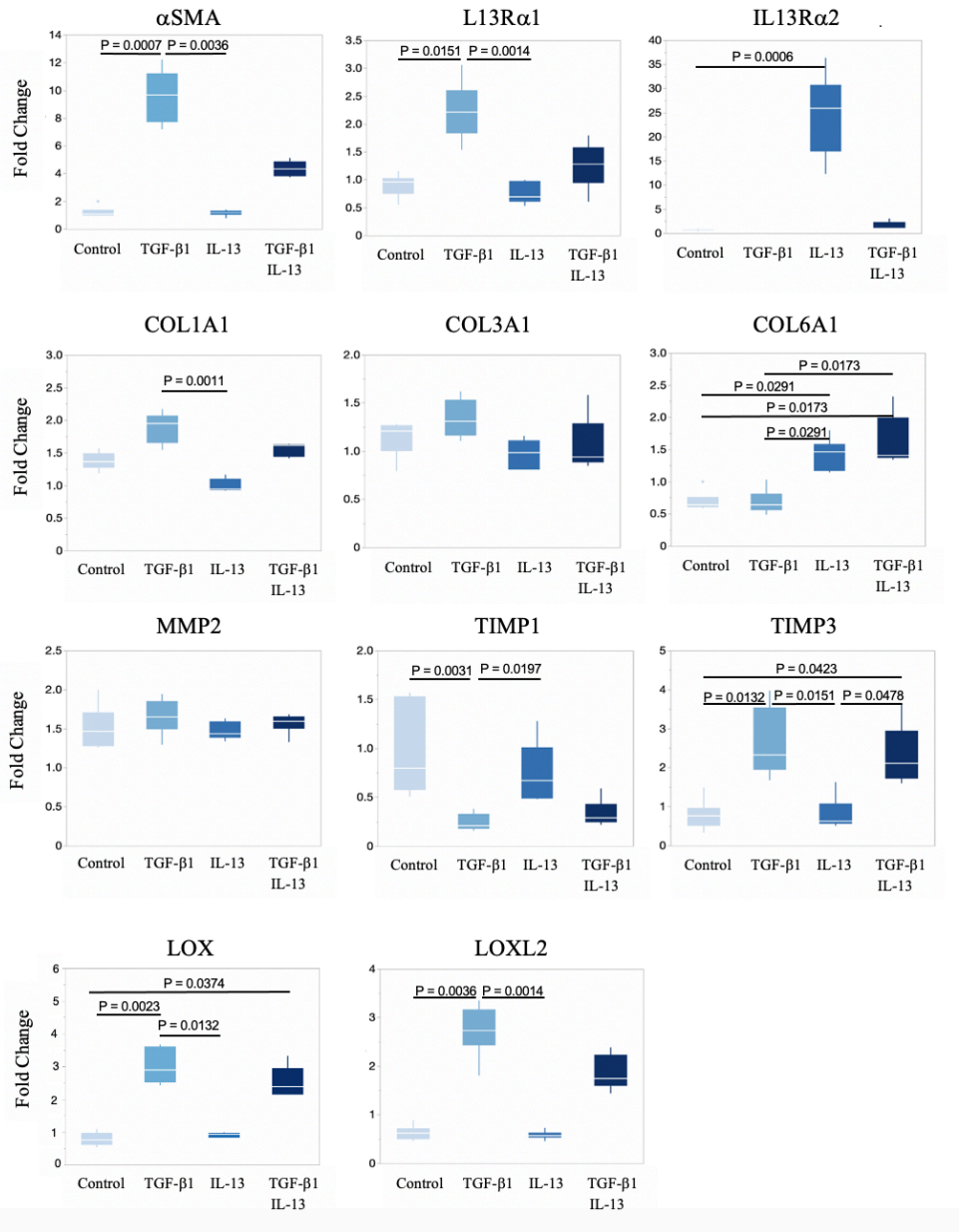

Figure 2.6: Gene expression is differentially regulated by treatment. Control and treated rings were snap frozen at days 7, 14, and 21, total RNA isolated, and mRNA levels of selected genes were quantified by RT-qPCR. Using the ΔΔCT method, levels at day 21 were compared to control values at day 7. Data for the entire time course are also presented (Supplemental Table 1). αSMA and IL13Rα1 are induced by TGF-β1, whereas IL-13 alone has no effect except when combined with TGF-β1 where it suppresses the induction. In contrast, IL13Rα2 is not expressed by TGF-β1 treated tissues, but it is greatly induced by IL-13. Interestingly, the combination of TGF-β1 and IL-13 suppresses the induction to control levels. COL1A1 and COL3A1 are unchanged or have minor changes with treatment, whereas COL6A1 is increased by IL-13 and TGF-β1 plus IL-13. MMP2 is unchanged by treatment. TIMP 1 is slightly suppressed by TGF-β1 and TGF-β1 plus IL-13. In contrast, TIMP3 is induced by TGF-β1 and TGF-β1 plus IL-13. LOX and LOXL2 are induced by TGF-β1 and TGF-β1 plus IL-13, with LOXL2 slightly lower in the combination treatment. (Kruskal-Wallis with post-hoc Dunn's test, $p<0.05$. n = 6).

and 2.7 fold, respectively and by TGF-β1 plus IL-13, 2.5 and 1.9 fold, respectively.

2.5 Discussion

TGF-β1 is widely accepted as a key mediator of fibrosis due to its well-known actions on fibroblasts, the cell type responsible for the excessive production of the collagenous matrix that compromises organ function, often by altering its biomechanics [5]–[8]. Here, we investigated TGF-β1's long-term action on human fibroblasts as they self-assemble and mature a collagenous 3D ring tissue over three weeks *in vitro*, a time-frame comparable to two of the three phases of wound healing [9]. This three week period was chosen based off of previous work in our laboratory finding maximization of biomechanics at this timepoint [26]. Furthermore, in lieu of a dose response, doses intended to maximize the fibrotic effects of the cytokines was chosen from the literature [27], [28]. We found that collagen levels and biomechanics of TGF-β1 rings increased rapidly by day 7, but stalled and declined slightly by day 21. However, when TGF-β1 was combined with IL-13, a cytokine exclusive to the immune system, levels of collagen and biomechanics continued to increase over three weeks. It's important to note that IL-13 alone was not able to mediate these effects, nor was TGF-β1 alone. These data suggests that TGF-β1 is able to induce a burst of collagen production and a quick rise in biomechanics, functions that IL-13 alone is not able to perform, but TGF-β1 is not able to continue the increase in collagen and biomechanics without the actions of IL-13. Thus, the combination of cytokines have complementary functions leading to a sustained increase in collagen levels and biomechanics over three weeks.

The stimulation of fibroblasts to synthesize collagen is a well know activity of TGF-β1 [10], [20], [29]. Less clear are the activities that are limiting the ability of TGF-β1 stimulated fibroblasts to sustain increasing collagen levels over three-weeks. Equally unclear are the activities provided by IL-13 signaling that enable TGF-β1 stimulated fibroblasts to sustain increasing collagen levels and biomechanics. Levels of collagen are regulated by processes that occur inside as well as outside the cell including transcription, translation, secretion, processing, assembly and turnover [30], [31], and so there are multiple points whereby IL-13 could potentially mediate its effects.

Although it is well known that TGF-β1 increases expression of the genes encoding the fibrillary collagens type I and type III [18], [29], [32], our gene expression data showed that at days 7, 14 and 21 there were little if any change in the levels of mRNA encoding COL1A1 or COL3A1 between control, TGF-β1, IL-13 or TGF-β1 plus IL-13 treated tissues. These suggest that IL-13 does not mediate its effects by upregulating these genes. In light of TGF-β1's documented ability to induce collagen gene expression, these results were surprising. We ruled out the possibility that fibroblasts were refractory and no longer responsive to TGF-β1 or IL-13 by assessing the levels of other genes known to be under their regulatory control. At day 21, TGF-β1 stimulated a 9.6 fold increase in αSMA and IL-13 stimulated a 24.6 fold increase in IL13Rα2, the decoy receptor for IL-13.

Collagen turnover is an important point of control that is highly regulated. Once synthesized, collagen has a long half-life, on the order of 70 days *in vitro* [33] and between 15 and 117 years *in vivo*, depending on tissue of origin [34]. Turnover is mediated by the matrix metalloproteinase (MMP) family of enzymes that vary in their specificity, cellular location, and

are typically synthesized in an inactive form [35], [36]. Thus, levels of MMPs, the proteolytic activators of MMPs, and the tissue inhibitors of matrix metalloproteinase (TIMPs) are all potential points of control.

The majority of collagen synthesized by our rings, control and treated, occurs within the first seven days, with only incremental increases at days 14 and 21. This is true for rings treated with TGF-β1, as well as TGF-β1 plus IL-13, that both synthesized nearly 3-fold more collagen than controls within the first week. Although we have not measured collagen half-life in our system, collagen is likely accumulating due to the steady increase in biomechanics and the maturation of fibers as seen in our SHG images. However, it is interesting to note, that there is no increase in collagen of TGF-β1 rings from day 7 to day 14, and collagen levels decrease from day 14 to day 21. This suggests that collagen turnover might be increased in the presence of TGF-β1, and that the combination of TGF-β1 and IL-13 could downregulate this turnover. It would be interesting to determine if collagen half-life is increased in TGF-β1 rings versus control rings, and decreased when TGF-β1 is combined with IL-13.

One collagen gene that is differentially regulated by IL-13 is collagen type VI, an interstitial collagen [37]. At day 21, IL-13 stimulates a modest, but significant increase in the mRNA encoding *COL6A1* over control ($P = 0.0291$) and TGF-β1 ($P = 0.0291$) rings. Unlike the fibrillary collagens types I and III, collagen type VI belongs to the class of network-forming collagens that are thought to produce more interconnecting fibrils rather than structural fibers [37], [38]. It's unclear if increased levels of collagen type VI are contributing to an increase in biomechanics and an alteration to the organization of collagen fibers as seen in the SHG images.

It would be interesting to determine at the protein level if TGF-β1 and IL-13 alter the ratios of the different collagen types and if this correlates with changes to biomechanics.

The cross sectional area of rings decreases over time as the tissues compact, and histology shows that cellularity decreases as the collagen matrix increases. At day 7, TGF-β1 rings and TGF-β1 plus IL-13 rings are larger than control and IL-13 rings, suggesting that TGF-β1 could be stimulating cell proliferation in the first week, but by day 21, TGF-β1 rings are no different than controls, whereas TGF-β1 plus IL-13 retain their larger size. Pyknotic nuclei were present in all tissues, but appeared to be present at a much greater extent in TGF-β1 and TGF-β1 plus IL-13 rings. It is interesting to note that pyknotic nuclei are present in the center as well as the outer edges of the tissue often adjacent to normal nuclei, ruling out the possibility that all cell death is simply due to diffusion limitations of the 3D tissue. Instead, this data suggests that as fibroblasts create the collagenous tissue, they undergo a process of terminal differentiation and that TGF-β1 may accelerate this process.

Multiple studies have investigated the complex and sometimes conflicting interplay of TGF-β1 and IL-13 *in vitro*, *in vivo,* and in clinical samples. In one study, non-fibrotic fibroblasts were responsive to TGF-β1, but not IL-13, whereas pro-fibrotic fibroblasts were hyper responsive to both [28]. IL-13 augmented TGF-β1's induction of TIMP1 in human airway fibroblasts, and the combined treatment of TGF-β1 and IL-13 also increased production of eotaxin-1, an autocrine inducer of collagen production [39]. TIMP1 expression in airway fibroblasts is reliant upon SMAD2/3 phosphorylation, and IL-13 augments this action in response to TGF-β1 [27]. IL-13 knock out (KO) mice failed to develop fibrosis after chronic infection despite the production of high levels of TGF-β1 [40]. Similarly, TGF-β1 KO mice

experienced a 20- to 50- fold increase in collagen I and III expression when treated with IL-13 [40]. These studies suggest an IL-13 dependent, TGF-β1 independent pathway for fibrosis. While our data does not directly suggest that IL-13 alone has a significant effect on inducing fibrotic properties in our ring tissues, it does suggest that TGF-β1 alone is insufficient to sustain profibrotic activity such as collagen deposition and increased tissue biomechanics and somehow relies on IL-13 to do so. Likewise, the association of TGF-β1 with IL-13 has been observed in clinical samples. Fibrotic tissue surrounds the lymph nodes of patients with classical Hodgkin lymphoma (CHL). Expression of TGF-β1 and IL-13 are detected in the neoplastic cells as well as the infiltrating mast cells, and increasing numbers of mast cells and expression of IL-13 correlated positively with higher rates of fibrosis [41][42]. Likewise, IL-13 expressing mast cells are increased in nodules from Dupuytren's contracture [43].

In our model, we simultaneously observe a decline in collagen content and biomechanics of TGF-β1 treated tissues, and a synergistic increase in these metrics in tissues treated with both TGF-β1 and IL-13. In attempting to explain these conflicting observations, we cannot rely solely on gene expression levels, particularly those of fibrillar collagens I and III which are not differentially expressed in our system. However, the discrepancy between gene expression and translated protein levels are widely understood [44], [45], and so it is possible that collagen is being translated at different rates and amounts than what is suggested by the PCR data. As such, it is also possible that a closer examination of mediators of remodeling and ECM modification such as MMPs, TIMPs, and LOX(L)s on the protein or enzymatic level would reveal a number of differences also not captured by gene expression levels. Likewise, it is also possible that changes in gene expression are occurring at time points earlier than we have examined, prior to day 7. We hypothesize a combination of these factors explain our observations, whereby ECM

degradation by active MMPs leads to decreased collagen and biomechanics for TGF-β1 treated tissues. In contrast, inhibition of remodeling by TIMPs, increased collagen synthesis, and post translational changes such as increased crosslinking could be responsible for increased collagen and biomechanics in ring tissues treated with both TGF-β1 and IL-13.

The results with TGF-β1 and IL-13 presented here demonstrate that ring tissues fill an important gap in the efforts to understand the mechanisms driving fibroblast dysregulation. Most *in vitro* studies use fibroblasts cultured as 2D monolayers and measure their effects 48 to 72 hours after treatment. The circular agarose molds in which ring tissues are formed provides a stable long-term *in vitro* environment for at least three to four weeks, a time frame spanning a major portion of the wound healing response *in vivo*. During this time, fibroblasts synthesize *de novo* a matrix rich 3D tissue with circumferentially aligned collagen fibers that can be examined by histology and has measurable mechanical properties. The ability to measure the impact of growth factor treatment on the strength and stiffness of ring tissues provides an important metric that links this *in vitro* model to the pathology of fibrotic organs. Although the model lacks the biological complexity of animal models, its focus is on the human fibroblast. The model provides a well-defined platform to dissect the complex autocrine (e.g., TGF-β1), paracrine (e.g., IL-13) and combination signaling that cause fibroblast dysregulation. Future studies can investigate the mechanisms by which TGF-β1 fails to sustain collagen accumulation and increases in tissue strength and stiffness, and how IL-13 sustains TGF-β1's actions.

2.6 Acknowledgements

The authors would like to thank Vera Fonesca for help with the biochemical assays. We thank the Leduc Bioimaging Facility for their support and especially Geoff Williams for assistance in SHG imaging and slide digitization and David Silverberg for IHC staining.

2.7 Supplemental Figures

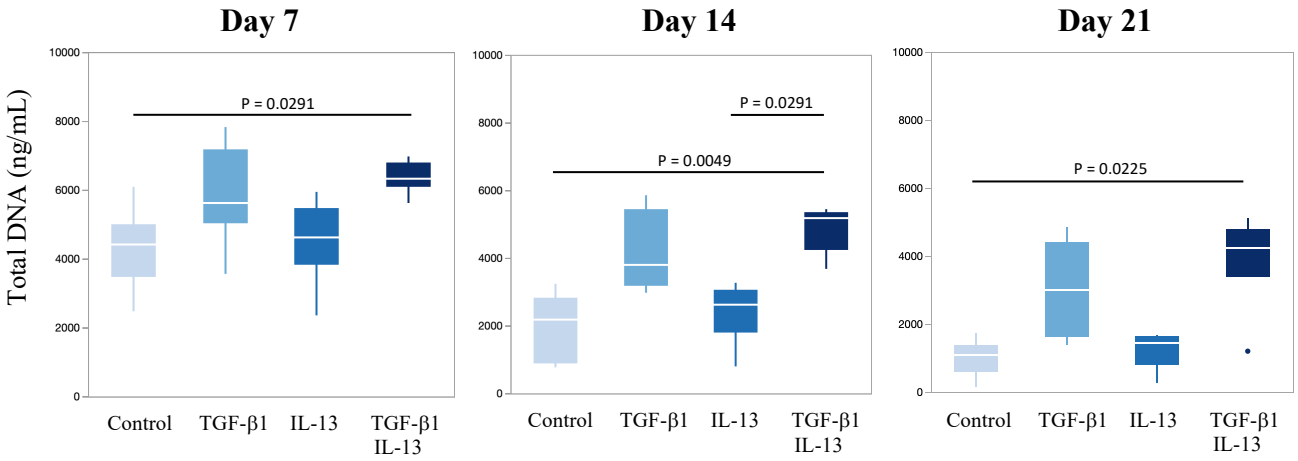

Figure 2.7: Control rings and rings treated with TGF-β1, IL-13 or TGF-β1 plus IL-13 were harvested at days 7, 14, and 21 and used to measure DNA. Levels of DNA varied with time and treatment. At day 7, the DNA content of rings treated with TGF-β1 or TGF-β1 plus IL-13 were significantly higher than control rings and rings treated with IL-13. Although DNA decreased for all conditions at days 14 and 21, the DNA content of rings treated with TGF-β1 or TGF-β1 plus IL-13 were considerably higher than control rings or rings treated with IL-13. (Kruskal-Wallis with post-hoc Dunn's test, $p<0.05$. n = 10).

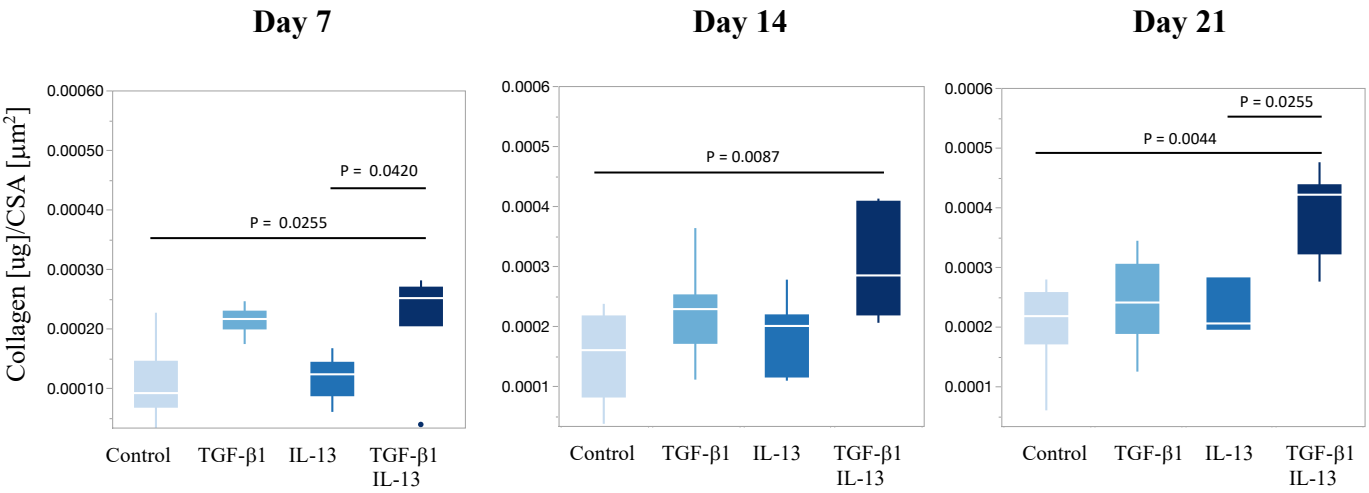

Figure 2.8: TGF-β1 in conjunction with IL-13 increases collagen density. Collagen density was calculated by dividing the total collagen content of each ring to each ring's cross sectional area (CSA). TGF-β1 increases collagen density as early as day 7, whether alone or with IL-13. Control rings and rings treated with IL-13 continue to moderately increase their collagen density over 14 and 21 days to levels similar to tissues treated with TGF-β1 alone. Interestingly, rings treated with both TGF-β1 and IL-13 continued to increase their collagen density over 21 days to levels significantly greater than control or IL-13 tissues. (Kruskal-Wallis with post-hoc Dunn's test, $p<0.05$. n = 10).

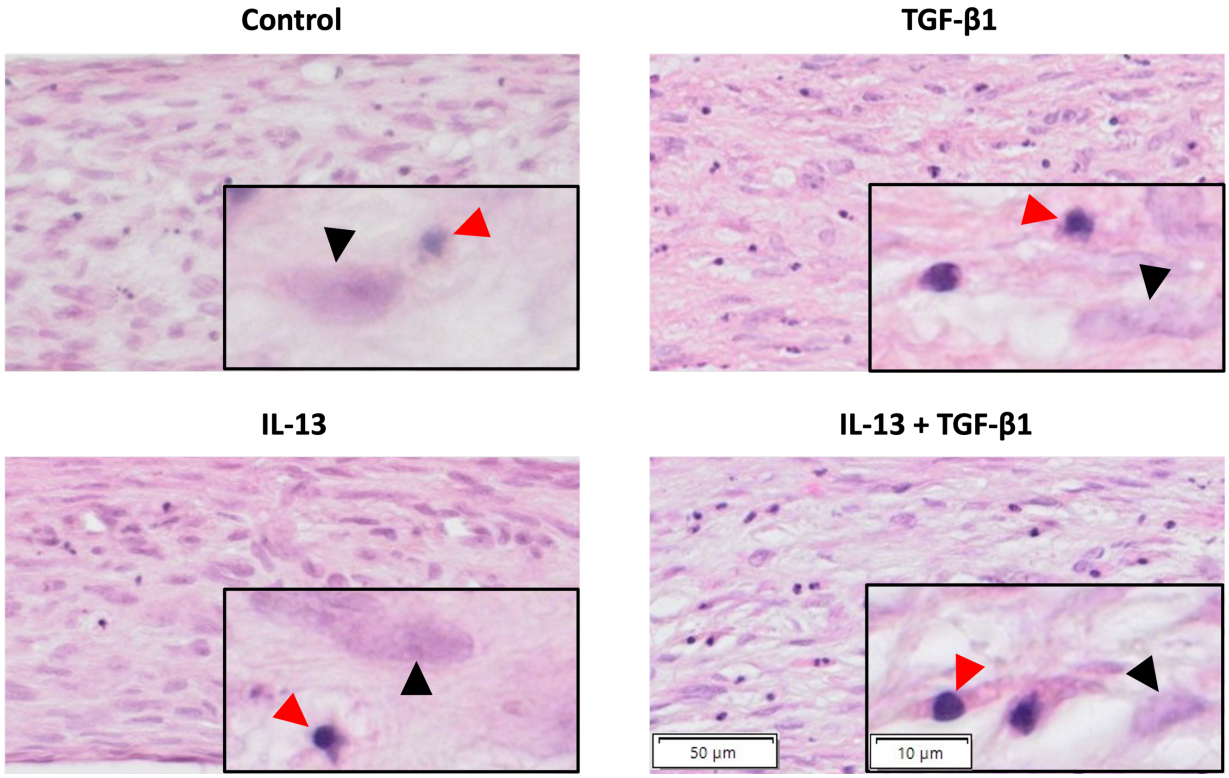

Figure 2.9: Treatment with TGF-β1 increases the presence of pyknotic nuclei. Histological images for control, TGF-β1, IL-13 and TGF-β1 plus IL-13 treated tissues at day 7 were acquired at 10x and 40x. Pyknotic nuclei were present in tissues from all conditions, but appeared to be increased in number in tissues treated with TGF-β1, whether alone or with IL-13. Higher magnification (40x) insets are here utilized to differentiate pyknotic nuclei (indicated by red arrow head) from healthy nuclei (indicated by black arrow head). Scale bars = 50 µm for original 10x images, and 10 µm for 40x insets.

2.8 Supplemental Tables

Sequence Name	Sequence (5' to 3')
COL1A1 FWD	GATTCCCTGGACCTAAAGGTGC
COL1A1 RVS	AGCCTCTCCATCTTTGCCAGCA
COL3A1 FWD	TGGTCTGCAAGGAATGCCTGGA
COL3A1 RVS	TCTTTCCCTGGGACACCATCAG
COL6A1 FWD	GCCTTCCTGAAGAATGTCACCG
COL6A1 RVS	TCCAGCAGGATGGTGATGTCAG
αSMA FWD	CTATGCCTCTGGACGCACAACT
αSMA RVS	CAGATCCAGACGCATGATGGCA
MMP2 FWD	AGCGAGTGGATGCCGCCTTTAA
MMP2 RVS	CATTCCAGGCATCTGCGATGAG
MMP9 FWD	GCCACTACTGTGCCTTTGAGTC
MMP9 RVS	CCCTCAGAGAATCGCCAGTACT
TIMP1 FWD	GGAGAGTGTCTGCGGATACTTC
TIMP1 RVS	GCAGGTAGTGATGTGCAAGAGTC
TIMP3 FWD	TACCGAGGCTTCACCAAGATGC
TIMP3 RVS	CATCTTGCCATCATAGACGCGAC
LOX FWD	GATACGGCACTGGCTACTTCCA
LOX RVS	GCCAGACAGTTTTCCTCCGCC
LOXL2 FWD	TGACTGCAAGCACACGGAGGAT
LOXL2 RVS	TCCGAATGTCCTCCACCTGGAT
IL13Rα1 FWD	CCTGAATGAGAGGATTTGTCTGC
IL13Rα1 RVS	CAGTCACAGCAGACTCAGGATC
IL13Rα2 FWD	GTGGAGTGATAAACAATGCTGGG
IL13Rα2 RVS	TGGGTAGGTGTTTGGCTTACGC
GAPDH FWD	GTCTCCTCTGACTTCAACAGCG
GAPDH RVS	ACCACCCTGTTGCTGTAGCCAA

Table 2.1. List of primers used for RT-qPCR of selected human genes using sequences obtained from OriGene.

	αSMA					IL13Rα1			
	Control	TGF-β1	IL-13	TGF-β1 + IL-13		Control	TGF-β1	IL-13	TGF-β1 + IL-13
Day 7	1.0	4.7	0.8	2.8	*Day 7*	1.0	2.0	0.9	1.0
Day 14	1.2	9.4	1.1	4.5	*Day 14*	1.1	2.3	1.0	1.5
Day 21	1.2	9.6	1.2	4.4	*Day 21*	0.9	2.2	0.8	1.3
	IL13Rα2					**COL1A1**			
	Control	TGF-β1	IL-13	TGF-β1 + IL-13		Control	TGF-β1	IL-13	TGF-β1 + IL-13
Day 7	1.0	n/a	14.6	0.3	*Day 7*	1.0	0.8	0.8	0.7
Day 14	1.2	n/a	24.5	0.7	*Day 14*	1.3	1.3	1.0	1.0
Day 21	0.6	n/a	24.6	1.5	*Day 21*	1.4	1.9	1.0	1.5
	COL3A1					**COL6A1**			
	Control	TGF-β1	IL-13	TGF-β1 + IL-13		Control	TGF-β1	IL-13	TGF-β1 + IL-13
Day 7	1.0	0.6	0.7	0.4	*Day 7*	1.0	0.6	1.6	1.1
Day 14	1.4	1.1	1.0	0.6	*Day 14*	0.8	0.6	1.8	1.4
Day 21	1.1	1.3	1.0	1.1	*Day 21*	0.7	0.7	1.4	1.6
	MMP2					**TIMP1**			
	Control	TGF-β1	IL-13	TGF-β1 + IL-13		Control	TGF-β1	IL-13	TGF-β1 + IL-13
Day 7	1.0	0.9	0.8	0.6	*Day 7*	1.0	0.4	0.8	0.4
Day 14	1.6	1.5	1.4	1.1	*Day 14*	1.0	0.2	0.9	0.4
Day 21	1.5	1.7	1.5	1.6	*Day 21*	1.0	0.2	0.8	0.3
	TIMP3					**LOX**			
	Control	TGF-β1	IL-13	TGF-β1 + IL-13		Control	TGF-β1	IL-13	TGF-β1 + IL-13
Day 7	1.0	4.0	0.7	2.7	*Day 7*	1.0	1.5	1.0	1.0
Day 14	1.0	3.3	0.9	2.8	*Day 14*	0.9	2.3	1.0	1.9
Day 21	0.8	2.6	0.8	2.3	*Day 21*	0.8	3.0	0.9	2.5
	LOXL2								
	Control	TGF-β1	IL-13	TGF-β1 + IL-13	*Fold change relative to Day 7 Controls. N/A denotes lack of expression under certain conditions.*				
Day 7	1.0	2.3	0.7	1.7					
Day 14	0.8	2.8	0.7	2.1					
Day 21	0.6	2.7	0.6	1.9					

Table 2.2: Gene expression is differentially regulated by treatment over the entire 3 weeks. Control rings and rings treated with TGF-β1, IL-13 or TGF-β1 plus IL-13 were snap frozen at days 7, 14, and 21, total RNA isolated, and mRNA levels of selected genes were quantified by RT-qPCR. Using the ΔΔCT method, levels at each week were compared to the control values of that same week.

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Chapter 3

Conclusion and Future Directions

Disease models are useful for both screening of molecules that have the potential to be effective therapeutics, as well as to better characterize disease development and characteristics on the molecular and cellular level. Many models have a number of shortcomings, however, such that they are not fully predictive of what is occurring *in vivo*. In addition to the complexity, expense, and ethical considerations that come with the use of animals in research, substantial discrepancies exist between the physiology and metabolism of human and animals, often rendering such trials unsuitable. Current *in vitro* models are a compromise between the complexity of the model and ability to be high throughput. Towards that end, this dissertation developed a more predictive model that recapitulates the fibrotic state *in vitro*.

Ways in which *in vitro* models in general can be more predictive include the use of genetically stable human cells in 3D, better recapitulating tissue physiology. For models of fibrosis specifically, there is often little focus on tissue mechanics, which is what ultimately determines organ function, given the hallmark stiffening seen in affected organs. In our determination to develop a more predictive *in vitro* model of fibrosis, we focused our efforts on three main objectives: (1) elucidate how soluble immune components TGF-β1 and IL-13 alter the biomechanical properties of an *in vitro* 3D ring tissue model to induce a fibrotic phenotype; (2) quantitatively and qualitatively characterize ring tissues treated with inflammatory TGF-β1 and IL-13 in order to understand what changes are occurring temporally, as determined by

collagen and DNA content analysis, histology, and second harmonic generation imaging; (3) determine the molecular mechanisms by which the combination of TGF-β1 and IL-13 facilitates the development of a fibrotic phenotype in a 3D ring tissue fibrosis model, using gene expression analysis.

Chapter 1 provides a comprehensive background of the landscape, including the structural and functional properties and capabilities of the extracellular matrix, as well as the purpose and process of wound healing. Both of those concepts are critical to understand prior to delving into the fibrotic disease state. This chapter also discusses the main effector cells of fibrosis, the fibroblasts, as well as the specific immune system components chosen to induce a fibrotic phenotype in our 3D ring tissues. Currently used *in vivo* and *in vitro* models were presented as a frame of reference.

Chapter 2 contains the experiments executed and data collected in the achievement of the specific aims of this project. We introduced soluble immune components TGF-β1 and IL-13 in our culture environment to induce a fibrotic phenotype. We characterized the strength and stiffness of these tissues utilizing tensile testing, leading us to observe a synergistic interaction between TGF-β1 and IL-13, as well as an unexpected attenuation in the strength of tissues treated with TGF-β1 alone. To discern the mechanism by which this occurred, we looked at collagen content and tissue architecture over time. To understand the molecular interactions driving these changes, we examined the expression of a number of genes of interest via PCR.

The outcome of this work is an improved *in vitro* model by which different cytokines and growth factors can induce an inflammatory environment suitable for the facilitation of the development of fibrosis. This platform utilizes solely human materials, has a long life in culture, and has measurable biomechanical properties informative of organ function. The model may be

used to investigate potential stimuli of fibrotic disease in a cause and effect fashion, as well as begin to discern the molecular mechanisms by which this disease occurs. This model can be used for screening of molecules of interest, as well as to test the efficacy of potential therapeutics, of which there is a great need. In the endeavor of finding new drugs to combat fibrosis, this model can be of use in conjunction with or in lieu of animals to facilitate greater productivity and a more efficient discovery process. While this work is valuable, there is still more to be done and other ideas to be considered.

3.1 Discussion

3.1.1 Changes in tissue architecture cannot be explained by proliferation or apoptosis

Observations of the CSA of tissues prior to mechanical testing allowed us to note changes in tissue size under various conditions. Tissues treated with TGF-β1, either alone or with IL-13, had a larger CSA than their control or IL-13 tissue counterparts at day 7. While the CSA of the other conditions declined over time, tissues treated with the combination of TGF-β1 and IL-13 had a significantly larger CSA than all other tissues at day 21. Alterations in tissue volume may be due to a number of changes, including increases in cell number, cell volume, or deposition of structural matrix proteins such as collagen. To investigate whether the change in tissue size was due to proliferation, IHC staining was performed for Ki67 (**Figure 3.1)**. Sparse staining was noted in all tissues at all time points, but particularly in tissues treated with TGF-β1, either alone or with IL-13, at day 7. However, the number does not appear to be robust enough to explain the significant size changes observed.

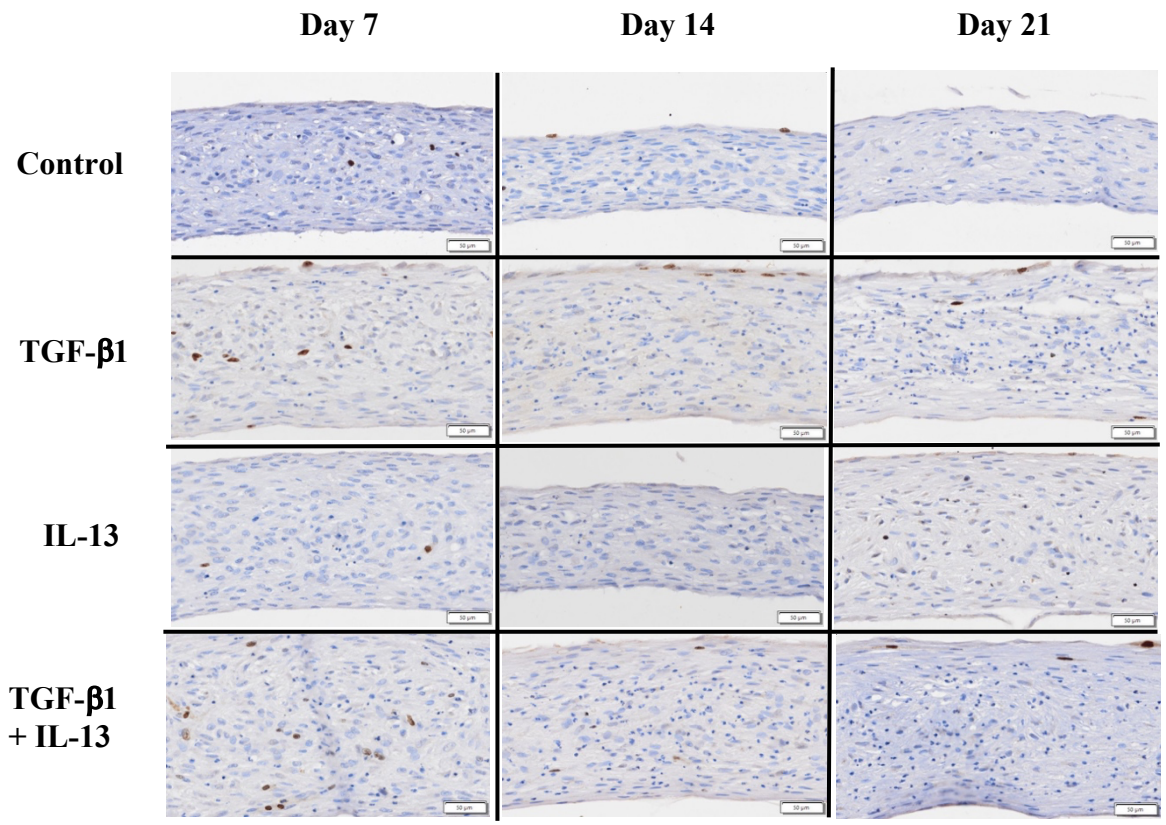


Figure 3.1: Cell proliferation is increased by TGF-β1. Control and treated rings were fixed, paraffin embedded, and proliferating cells identified by immunostaining for Ki67. Stained sections were imaged at 10X magnification using the Olympus VS200 Slide Scanner. The bottom side of each section is the surface of the ring tissue that contacts the agarose peg. Small numbers of Ki-67 positive proliferating cells were identified in all rings and their numbers decreased with time in culture. Proliferating cells were identified in the center of the rings as well as the outer edge of the tissues. Compared to control and IL-13 treatment, rings treated with TGF-β1 alone or TGF-β1 plus IL-13 had increased numbers of Ki-67 positive cells, particularly at day 7. Scale bars= 50 μm.

Similarly, when histology was performed, a large number of pyknotic nuclei were observed in all tissues, increasing in number over time, with the greatest amount appearing to be in TGF-β1 and TGF-β1 and IL-13 treated tissues. To determine if cells were dying due to apoptosis, IHC staining for TUNEL was performed (**Figure 3.2**). While TUNEL positive nuclei were identified in all conditions at most time points, the prevalence was sparse, and there were

far more pyknotic nuclei present than apoptotic nuclei, indicating that another mechanism of cell death is likely responsible for the presence of the pyknotic nuclei. It is possible that the cells succumbed to necrosis, due to limitations of nutrient diffusion in larger tissues, but we consider this to be unlikely due to the fact that healthy nuclei are immediately adjacent to pyknotic nuclei in the center of all tissues, and that pyknotic nuclei are present in all tissues regardless of thickness.

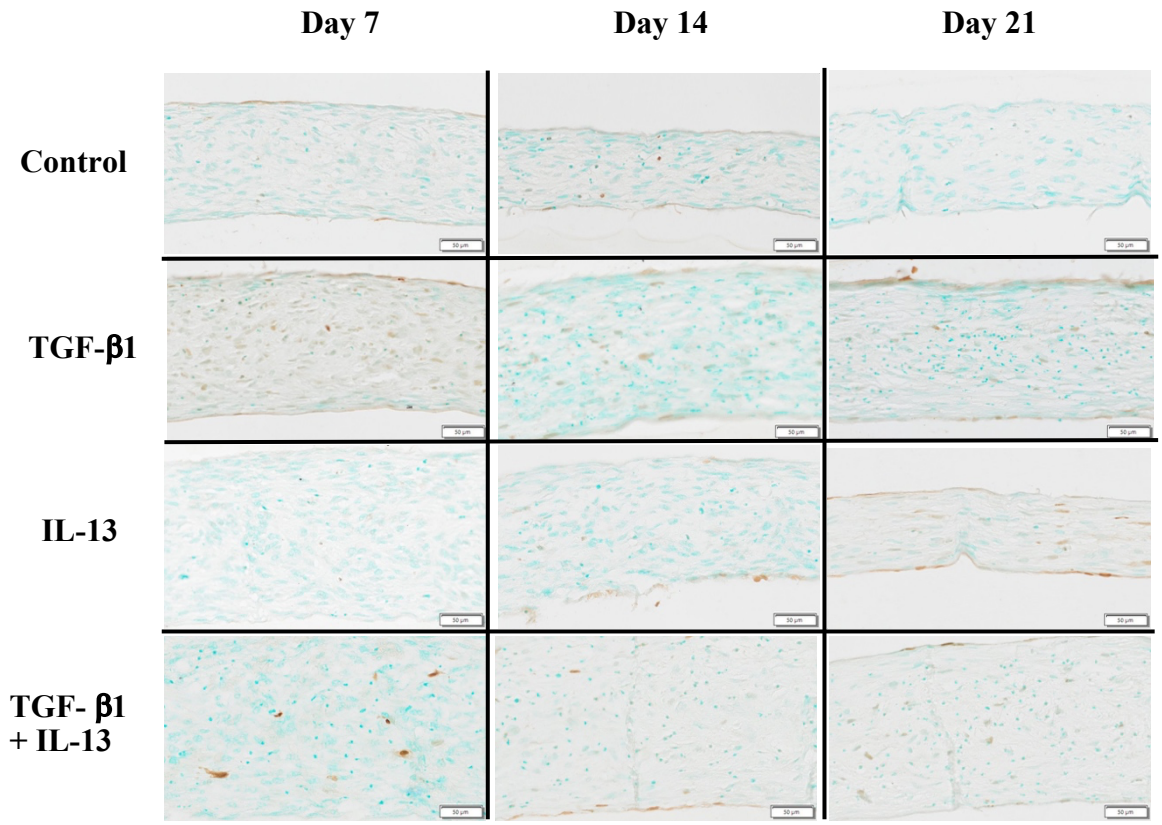


Figure 3.2: Ring tissues have a high level of pyknotic and low level of apoptotic nuclei seen across all conditions. Control and treated rings were fixed, paraffin embedded, and subjected to the TUNEL assay to identify apoptotic cells. Stained sections were imaged at 10X magnification using the Olympus VS200 Slide Scanner. The bottom side of each section is the surface of the ring tissue that contacts the agarose peg. Small numbers of TUNEL positive cells were identified in all conditions across almost all time points, both in the middle of the tissue and at the periphery. TUNEL positive cells were increased in rings treated rings treated with TGF-β1 alone or TGF-β1 plus IL-13. Interestingly, pyknotic nuclei far outnumbered TUNEL positive cells. Scale bars= 50 μm.

However, it is important to note that while TUNEL is highly advertised as a stain capable of distinguishing apoptotic cells, in reality, the assay labels all free 3'-hydroxyl termini, simply indicating the presence of DNA damage, which is not unique to the process of apoptosis [1]. Thus, it is possible that the cells in question did not die via mechanisms of cell death resulting in DNA fragmentation. It has been demonstrated in the literature that the incidence of TUNEL-negative pyknotic nuclei has been previously observed in bovine granulosa cells; in this instance, it was concluded that their death was more consistent with that as a result of terminal differentiation, such as what occurs during keratinization of the skin [2]. Similarly, epithelial cells in the lens of the eye undergo a terminal differentiation process to fibers where pyknotic nuclei are observed [3]. Thus, it is important to consider that the dermal fibroblasts in our ring tissues may have undergone terminal differentiation. The mechanism by which cells die during the final stages of terminal differentiation is still unclear [4], as is the reason why it appears to be driven by treatment with TGF-β1 in our system.

3.1.2 Treatment with TGF-β1 fails to produce strong tissues over time

When we assessed both changes in biomechanics and collagen content over time and across conditions, we observed a series of trends. Control tissues and tissues treated with IL-13 demonstrated a slow and steady increase in collagen and mechanics over time. We've discussed at great lengths how tissues treated with both TGF-β1 and IL-13 experienced a significant increase in mechanics and collagen deposition early on, which was bolstered by continued increase for the duration of culture. Tissues treated with TGF-β1 alone, however, experienced a robust increase in collagen and mechanics at day 7, which attenuated such that by day 21, they

were weaker than control or IL-13 treated tissues. While the mechanism through which this occurs is unknown, there are a few possible explanations.

We know the cells are not becoming refractory to cytokine signaling at later time points, as evidenced by the PCR data showing differential expression of αSMA and IL13Rα1 in TGF-β1 treated tissues, as well as expression of IL13Rα2 in IL-13 treated tissues. While this may be true, it is well documented that desensitization of the ligand can occur over the course of long term TGF-β1 signaling. It is known that TGF-β1 is produced and acts on fibroblasts in both autocrine and paracrine fashions, and there is evidence for modulation of signaling depending on alternative stimulus, where cells with low levels of autocrine activity will respond robustly to acute paracrine signaling, but conversely, cells with high levels of autocrine activity will respond weakly to the same stimuli. Further, it has been suggested that in spite of the refractory state induced by long term exposure to high levels of TGF-β1, a low level of signaling is still maintained, allowing for the expression of some genes in response to TGF-β1 but silencing others that require a stronger signal [5]. This provides a possible explanation for how at later timepoints TGF-β1 is capable of inducing the expression of αSMA, but there is no differential expression of collagens I or III.

Alternatively, as previously touched upon, it is possible that the seemingly increased number of pyknotic nuclei observed in tissues treated with TGF-β1 simply leads to fewer cells participating in activities such as collagen synthesis and post translational modification of the matrix that would lead to the phenotype of increased tissue strength and stiffness over time. For this reason, it would be interesting to utilize a methodology to quantify the number of cells present at each time point under each treatment condition, such as a grid count. While tissues treated with TGF-β1, alone or with IL-13 appear to have a greater number of pyknotic nuclei, it

is solely tissues treated with TGF-β1 alone that experience the attenuation in the development of the fibrotic phenotype; something about the interaction of TGF-β1 with IL-13 allows for this limitation to be overcome. While the mechanism is unknown, a theoretical explanation is proposed below.

3.1.3 Implication of altered IL-13 receptor gene expression

The two receptors for IL-13, as well as the concept that one, IL-13Rα2, may serve as a decoy, was briefly touched upon in the previous chapters. Several studies have countered the idea of the decoy receptor, illustrating evidence that signaling through IL-13Rα2 activates the AP-1 transcription factor, subsequently activating the TGF-β1 promoter [6], [7]. Alternatively, other studies have demonstrated results that support the concept of a decoy, such that inhibition of IL-13Rα2 allows for increased signaling of IL-13, resulting in increased fibrosis [8], [9]. Because IL-13Rα2 has a much higher affinity for the ligand than IL-13Rα1, it would be theoretically possible for it to sequester any ligand present and prevent it from having downstream effects [10], [11].

When attempting to determine changes in gene expression as the result of cytokine treatment in our system, we did look for alterations in expression of IL-13 receptors. We found that IL-13 upregulated its own decoy receptor, over 14- fold at day 7 and nearly 25-fold at days 14 and 21, likely as a self-regulating negative feedback mechanism. What was interesting, however, was that the combination of TGF-β1 and IL-13 returned the levels of IL-13Rα2 back to baseline, with a maximum of 1.5-fold expression relative to control at day 21 (**Supplementary Table 2.2**). This indicates that the cells were not becoming refractory to the

long time course of high dose cytokine treatment, but it may additionally serve as an avenue to explain some of the changes seen in the combination treated tissues. Given how significantly IL-13 upregulated its own decoy receptor, it is very likely that with the high affinity of the receptor, that the majority of the ligand was sequestered and prevented from engaging in signaling that would induce a fibrotic phenotype. Conversely, if TGF-β1 assisted with the significantly decreased expression of the decoy receptor, it is possible that ligand was available to bind to the signaling receptor, IL-13Rα1, and participate in signaling that contributed to the robust fibrotic phenotype observed.

It would be tremendously valuable to attempt to validate this theory by neutralizing the decoy receptor, such as through the use of a neutralizing antibody, and subsequently treating with a high dose of IL-13. Assuming the theory presented is correct, one would expect to see a separate phenotype, where collagen content and biomechanics of tissues treated with IL-13 following receptor inhibition would be increased compared to IL-13 treated tissues with the receptor left intact. In our scenario, the fibrotic phenotype induced by combination treated tissues may be the result of a strong initial response provoked by TGF-β1, followed by continued collagen and strength and stiffness accumulation at the hands of IL-13 allowed to signal to its full potential.

3.1.4 Gene expression analysis fails to explain phenotypic changes and cytokine synergy

Examination of expression of selected genes did not reveal a clear mechanism explaining the phenotypic synergy observed in TGF-β1 and IL-13 treated tissues. Some genes were not differentially expressed (i.e. MMP2, COL3A1), some only under certain treatment conditions, and others still in a manner that does not explain the phenotypic changes in strength, stiffness,

and collagen deposition observed; for example, both TGF-β1 and TGF-β1 and IL-13 treated tissues upregulate expression of LOX and LOXL2, but only the combination treated tissues experience greater biomechanics and collagen content.

It is understood that mRNA expression levels do not necessarily correlate with protein product. In fact, there is typically only a 40% correlation between mRNA and protein genome wide. However, mRNAs that are differentially expressed correlate much better with their protein product than their non-differentially expressed counterpart genes [12]. Additionally, in understanding the central dogma, we know that there are a number of steps involved in the genetic flow of information from DNA to RNA to protein, after which there are a significant amount of post-translational modifications possible; proteins are also subject to degradation [13]. As a result, it is possible that the select gene expression profiles captured here truly are not representative of the protein products affecting phenotypic change in our tissues. Alternatively, it is also possible that the timescale under which the experiments were performed was unsuitable to observe the changes in mRNA expected; indeed, many of the published experiments informing the development of the study presented herein examine gene expression of 2D cell cultures that have been treated with our cytokines of interest for a mere 24-48 hour period [14]–[16].

The two differentially expressed mRNAs that could possibly explain some of the phenotypic changes observed are IL13Rα2, which was previously discussed, and COL6A1. Collagen VI is the most comprehensively studied member of the family of beaded-filament-forming collagens, which are widespread in tissues and interact with many matrix components [17]. They are interspersed with collagens I and III and work to regulate fibril diameter and interconnect individual fibrils; they also play a role in hemostasis, which is indispensable in the early phases of wound healing and fibrotic responses [18]. Of note, collagen VI has been

implicated in the development of a fibrotic phenotype, and has been seen to be expressed in a co-localized manner with αSMA in fibrotic foci from patients with IPF and Dupuytren's Disease. It is routinely seen to be elevated in patients with a number of chronic liver and kidney diseases, and a fragment from its inactive precursor, endotrophin, is commonly used as a biomarker in the evaluation of disease progression [19]. However, collagen VI has also been proven to be essential for routine structure and function, as the matrix of skeletal muscle is heavily comprised of collagen VI. Interestingly, gene mutations involved in its formation have been implicated in the development of several myopathies and muscular dystrophies [20]. Whether the upregulation of gene expression in our system would lead to the phenotypic changes observed is uncertain, but it is clear that collagen VI is a target that should be further examined.

3.2 Future Directions

3.2.1 Tuneability of de novo synthesized matrix

One of the greatest advantages of the model developed is the inherent tunability. While we chose to look at the effects of TGF-β1 and IL-13 over the duration of 3 weeks on ring tissues formed from juvenile dermal fibroblasts, another investigator could theoretically change any of the aforementioned variables to suit the needs of the question they are trying to answer. In the past, we have run preliminary trials forming rings using fibroblasts of lung origin, and other members of the lab have formed 3D organoids of various geometries using a number of different cell types. As an example of the flexibility of the system, to initially determine optimal conditions for the growth of our dermal fibroblasts in a ring geometry, a study utilizing various

compositions of cell culture media was undertaken. At periodic intervals, the tissues were harvested and histology was performed to examine the tissue architecture and overall health. Dramatic differences were visualized between media conditions, and choices were made to go forward using a media that appeared to support the development of healthy, uniform tissues with a balanced interplay between cells and secreted matrix [21]. This process of media optimization could theoretically take place for any cell type of interest.

As discussed regarding gene expression analysis, many of the changes taking place may be occurring at much earlier timepoints than analyzed in this study. We know that our organoids form over the first 24 hours in culture, and studies can reasonably focus on any timepoints thereafter. We chose to introduce our proinflammatory cytokines of choice within the first 24 hours of culture, and utilized a high dose for the duration of culture, but a variety of dosing schemes could be utilized. A dose response could be performed to determine optimal concentration for desired effect with limited off target effects, or dosing could take place for a shorter timeframe with a subsequent period of recovery to examine effects under those conditions. Finally, we chose to look at the effect of canonical TGF-β1 and its interaction with IL-13, a signal that acts on fibroblasts but is secreted exclusively by the immune system, but numerous cytokines or growth factors could be chosen for study. For example, fibroblast growth factor (FGF), produced by macrophages, is capable of stimulating fibroblast proliferation, which may have interesting measurable effects. Alternatively, IFN-γ, which is produced by T cells, acts counter to TGF-β1, inhibiting collagen production and thus hypothetically decreasing biomechanics [22].

3.2.2 Quantification of SHG images

We performed multiphoton second harmonic generation imaging to get representative image stacks of our collagen matrix under various treatments over time. There is value in qualitatively assessing such z sacks, as observations can lead us to consider ideas worthy of pursuing from a quantitative perspective, such as whether the appearance of increased interconnected collagen filaments may indicate crosslinking (**Videos 3.1-3.4**). However, a limitation of the work presented herein is the lack of quantitative analysis on the image stacks obtained. It would be extremely interesting and informative to quantitatively assess fibril diameter, direction, and space between bundles using techniques such as Fourier transform. This would mathematically confirm observations that collagen fibril alignment generally tends to increase over time, but that certain conditions experience unique fibril architectures, potentially denoted by less alignment. Additionally, if collagen fibril diameter was seen to be increasing over time, in conjunction with increased density denoted by decreased interfibrillar space, there would be a possible explanation for the observed increased tissue strength and stiffness under certain treatments. A collaboration that leads to collection of such data would be extremely useful.

3.2.3 RNA-Seq may uncover alternative molecular pathways

An inherent flaw in the execution of studies involving techniques such as RT-qPCR is the biased selection of a limited number of genes of interest. Investigators can choose, in conjunction with informed guesses from the literature, which genes they believe will experience changes under the circumstances of the study, but it is possible, and in fact likely, that certain changes of

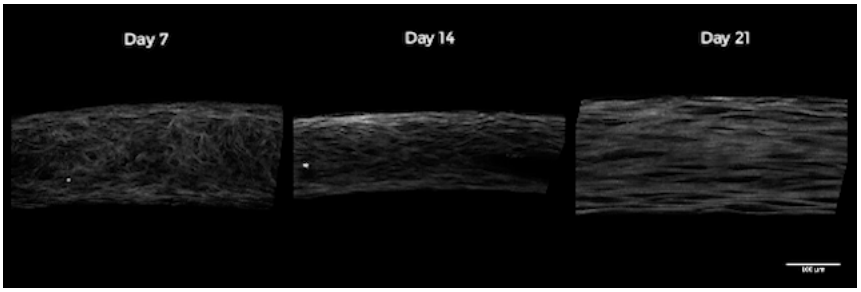
significance will be missed. In the case of this work, the expression levels for MMP1, or collagenase, should have been studied, but were overlooked. For that reason, it is becoming more common to utilize newer, more encompassing techniques to look at changes in gene expression level via Next Generation Sequencing. Techniques such as RNA-Seq have the ability to examine RNA expression across the entire genome, eliminating selection bias and providing a much greater scope and understanding of cellular function and activity. Samples collected in the course of this study have been sent to the University's genomic core facility for RNA-Seq analysis in the hopes of uncovering a mechanism that would explain some of the phenotypic findings observed. Regardless, it will provide a considerable amount of data that will inform future areas of study in our model. In the future, it would be beneficial to consider the use of proteomics to better determine tissue structure and function on the protein level.

3.3 Conclusion

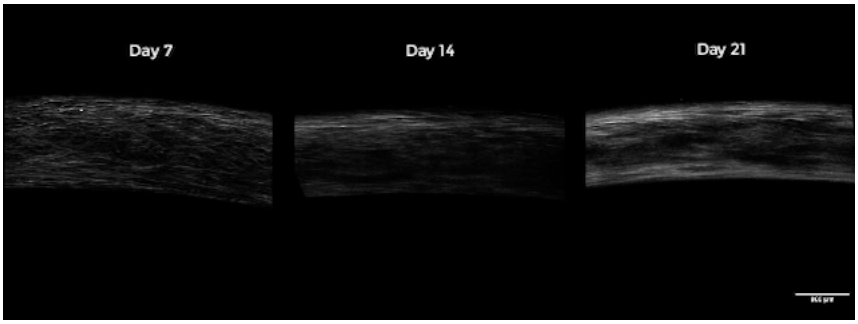
This study developed a predictive *in vitro* model for fibrosis by incorporating soluble immune factors into a three-dimensional ring tissue comprised of human cells. This model is capable of conveying information about tissue mechanics, collagen content, and biochemical alterations over time and across various treatment conditions. The combination of TGF-β1 and IL-13 resulted in the development of a fibrotic phenotype, evidenced by significantly increased tissue strength and stiffness, as well as collagen deposition, compared to controls or tissues treated with either cytokine alone. While the mechanism by which this occurs is not fully understood, a number of proposed means are capable of being further studied due to the usefulness of this highly tunable model.

3.4 Videos

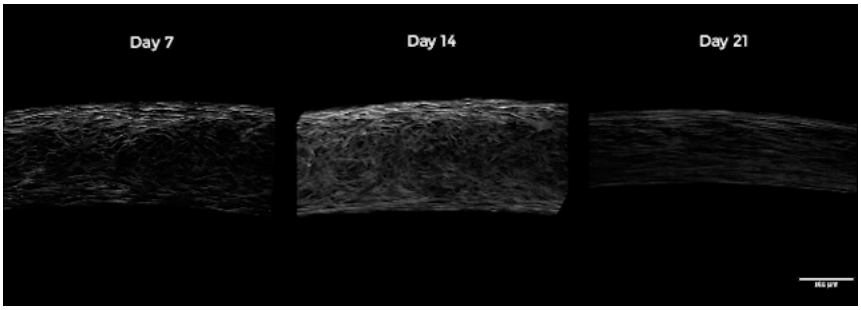
3.1: Control Tissues



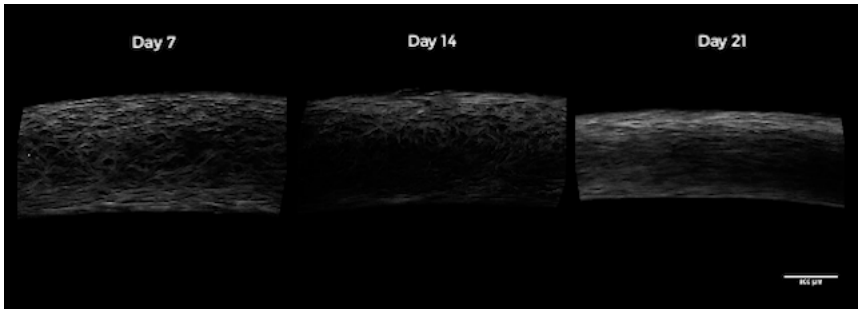
3.2: TGF-β1 Tissues



3.3: IL-13 Tissues



3.4: TGF-β1 + IL-13 Tissues



Videos 3.1-4. The 3D architecture of fibrillar collagen varies with time and treatment. Fibrillar collagen of control rings (1) and rings treated with TGF-β1(2), IL-13 (3) or TGF-β1 plus IL-13 (4) were imaged at days 7, 14, and 21 using multiphoton second-harmonic generation (SHG) microscopy. Rings were fixed in formalin, submerged in PBS, and imaged *in situ* within their agarose molds. The bottom side of each image is the surface of the ring tissue that contacts the agarose peg. Z stacks are captured from the inner region of the tissue moving outward. Scale bar = 100 μm. DOI: https://doi.org/10.7910/DVN/DGKRPV

3.5 References

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