Antifibrotic Effects of Vibratory Stimulation

Sooneon Bae
Clemson University, sooneon@gmail.com

Follow this and additional works at: https://tigerprints.clemson.edu/all_dissertations

Recommended Citation
https://tigerprints.clemson.edu/all_dissertations/1879

This Dissertation is brought to you for free and open access by the Dissertations at TigerPrints. It has been accepted for inclusion in All Dissertations by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
ANTIFIBROTIC EFFECTS OF VIBRATORY STIMULATION

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Sooneon Bae
May 2017

Accepted by:
Dr. Ken Webb, Committee Chair
Dr. Jeoung Soo Lee
Dr. Jeremy L. Barth
Dr. Jiro Nagatomi
ABSTRACT

Extracellular matrix (ECM) is a dynamic and complex environment characterized by biophysical, mechanical and biochemical properties specific for each tissue. Cells constantly experience dynamic mechanical loadings that include compression, shear, tension, hydrostatic pressure, and interstitial fluid flow. Through the process of mechano-chemical conversion, mechanical stimulation activates intracellular biochemical signaling that affects many aspects of cell behavior including cell proliferation and differentiation, as well as ECM deposition and organization during development, wound healing, and pathological diseases. Despite significant advances in understanding the dynamic relationship between mechanical forces and matrix remodeling, many of the unique mechanisms and associated responses to various physical stimuli remain to be elucidated.

Fibrosis is a complex disease predominantly characterized by excessive and abnormal fibrous ECM deposition that leads to the failure of various organs: lung, liver, kidney and skin. During the normal wound healing process, injured tissue progresses through phases of hemostasis, acute inflammation, granulation tissue/fibroproliferative, matrix formation, and remodeling. Collectively, the fibro-proliferative stage terminates with the restoration of ECM homeostasis and the disappearance of myofibroblasts, probably through apoptosis. However, the chronic presence of diverse injuries, commonly involving the abnormal persistence of several profibrotic cytokines results in sustained myofibroblast activation, excessive ECM deposition, scar formation, and organ failure. Specifically, transforming growth factor-β (TGF-β) is a master switch that activates critical downstream molecules in the progression of fibrotic disease. Although
various strategies designed to interfere with TGF-β expression, receptor binding, and signal transduction have been studied, a clinically safe and effective therapy has not yet been developed.

The superficial layer of the lamina propria (SLLP) in the human vocal folds experiences a unique mechanical microenvironment of high frequency vibration during voice production. The presence of macrophages/myofibroblasts in the SLLP of healthy patients suggests that the mechanical stresses imposed during routine speech result in repetitive microtrauma, which is generally repaired without permanent alterations in vocal fold matrix composition or vocal quality. In addition, mechanical forces have recently been shown to alter the fibrotic phenotype in fibrotic fibroblasts. Therefore, the objective of this research is to understand the mechanisms regulating fibroblast matrix metabolism in the SLLP and investigate the potential of vibratory stimulation for treatment of fibrotic diseases.

First, we characterized the transcriptional and translational changes of human dermal fibroblasts in response to vibratory stimulation and demonstrated that vibratory stimulation led to the down-regulation of the TGF-β signaling through reduced expression of TGF-β receptors and Smad signal transduction molecules and increased expression of SMAD7, ubiquitin ligases, and SIK1 and SKIL, transcriptional repressors responsible for signaling inhibition. Second, we then investigated the effects of variable vibratory regimes defined by varying frequency, amplitude, and duration on the expression of ECM-related transcripts in human dermal fibroblast and found significant dose-dependent and temporal changes in mRNA expression levels of HA-related
molecules and profibrotic cytokines, while type I and III collagen expression was consistently down-regulated across a broad range of parameters. Finally, we tested the potential therapeutic efficacy of vibration for reversing the fibrotic phenotype in scleroderma-derived fibroblasts. These studies showed that vibratory stimulation significantly reduced the mRNA levels of sclerotic pathogenic targets and collagen synthesis and accumulation. These studies, therefore, suggest that vibration can be used as a clinical mechanotherapy for a wide range of fibrotic diseases such as systemic sclerosis and idiopathic pulmonary fibrosis.
ACKNOWLEDGMENTS

I would like to thank you to all who have supported, encouraged, and helped me to achieve my doctoral degree at Clemson University. There is no way that I can adequately express how grateful I am to each individual in this short section. I am very thankful to my advisor, Dr. Ken Webb, for his continuous support and guidance during my graduate years. His expertise and insight for the research greatly impressed me and allowed me to expand my scientific knowledge of mechanobiology and mechanotherapy. Without his guidance, I would have never finished this dissertation. I would also like to thank Dr. Jeoung Soo Lee, who gave me an opportunity to start my journey in Bioengineering at Clemson University. Her passion and knowledge in polymer chemistry and synthesis greatly influences the way I approach research and helped me broaden my scientific skills, making me a better scientist. I am also appreciative to my committee members, Dr. Jiro Nagatomi and Dr. Jeremy L. Barth, who have guided me to finish my dissertation. I greatly appreciate their intellectual support and critical suggestions to improve the quality of my graduate training.

I would like to sincerely acknowledge numerous people who have helped me through this journey, some by their technical assistance and some by their friendship and moral support. These people include Maria Torres, Dr. Martin Laberge, Dr. So Jung Gwak, Dr. Da Un Jeong, Dr. Atanu Sen, Dr. Jeremy Zhang, Dr. Ho-Joon Lee, Linda Jenkins, Cassie Gregory, Chad L. McMahan, Leigh Humphries, Chris Macks, Prathik Thulluri, and Zackary Reinhardt.
Finally, and most importantly, I would like to express my deepest thanks to my wife, Jiyun Kim; my son, Brian Hyunseo Bae; and my daughter, Grace Sooyeon Bae, who have been with me all the time through my journey. I am also very grateful to my family living in South Korea: my father, Chilseok Bae; my mother, Kyoungsun Kim; my brother, Soonmin Bae; my father-in-law, Myungjoong Kim; and my mother-in-law, Youngsook Hwang. I am very thankful for their invaluable support, their extreme patience, their most appreciated help and efforts to make this journey so special. Without them, most of this work would not have been possible.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>....................................................................................................................</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>..................................................................................................................</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>........................................................................................................</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>..........................................................................................................</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>........................................................................................................</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER</td>
<td>I.  INTRODUCTION ..........................................................................................</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.1. Extracellular Matrix (ECM) ..............................................................</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.1.1. ECM Remodeling ...........................................................................</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.1.2. Regulation of Matrix Remodeling ...............................................</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.1.2.1. Growth Factors and Cytokines ..................................................</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.1.2.2. Mechanical Microenvironment ..................................................</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1.2. Wound Healing ..................................................................................</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1.2.1. Hemostasis ....................................................................................</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1.2.2. Inflammatory Phase ......................................................................</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1.2.3. Proliferative Phase ......................................................................</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>1.2.4. Remodeling Phase .........................................................................</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>1.3. Fibrosis ...........................................................................................</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1.3.1. TGF-β Signaling and Fibrotic Diseases ........................................</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>1.3.2. Reversibility of Fibrosis ............................................................</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>1.4. Current Antifibrotic Therapy and Limitation ....................................</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>1.4.1. Interfering with TGF-β Expression and Activation .........................</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>1.4.2. Blocking TGF-β Signaling Pathways including TGF-β receptors and SMAD molecules</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>1.5. Mechanotherapy ................................................................................</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>1.5.1. Mechanotransduction .....................................................................</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>1.5.2. Effect of Mechanical Stimulation on ECM Remodeling-related Molecules</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>1.5.3. Current Mechanotherapy in Wound Healing .....................................</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>1.6. Vocal folds .........................................................................................</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>1.6.1. Vocal Fold ECM and Biomechanics ................................................</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>1.6.2. Scarless Wound Healing of Vocal Fold ........................................</td>
<td>52</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>II.</td>
<td>PROJECT RATIONALE</td>
<td>55</td>
</tr>
<tr>
<td>2.1.</td>
<td>Specific Aims</td>
<td>58</td>
</tr>
<tr>
<td>2.2.</td>
<td>Significance and Innovation</td>
<td>61</td>
</tr>
<tr>
<td>III.</td>
<td>ANTIFIBROTIC EFFECTS OF VIBRATORY STIMULATION ON HUMAN FIBROBLASTS</td>
<td>61</td>
</tr>
<tr>
<td>3.1.</td>
<td>Abstract</td>
<td>61</td>
</tr>
<tr>
<td>3.2.</td>
<td>Introduction</td>
<td>62</td>
</tr>
<tr>
<td>3.3.</td>
<td>Materials and Methods</td>
<td>64</td>
</tr>
<tr>
<td>3.4.</td>
<td>Results</td>
<td>74</td>
</tr>
<tr>
<td>3.5.</td>
<td>Discussion</td>
<td>87</td>
</tr>
<tr>
<td>3.6.</td>
<td>Conclusion</td>
<td>91</td>
</tr>
<tr>
<td>IV.</td>
<td>THE EFFECT OF VARIABLE VIBRATORY PARAMETERS ON FIBROBLAST MATRIX SYNTHESIS</td>
<td>93</td>
</tr>
<tr>
<td>4.1.</td>
<td>Abstract</td>
<td>93</td>
</tr>
<tr>
<td>4.2.</td>
<td>Introduction</td>
<td>94</td>
</tr>
<tr>
<td>4.3.</td>
<td>Materials and Methods</td>
<td>97</td>
</tr>
<tr>
<td>4.4.</td>
<td>Results</td>
<td>102</td>
</tr>
<tr>
<td>4.5.</td>
<td>Discussion</td>
<td>111</td>
</tr>
<tr>
<td>4.6.</td>
<td>Conclusion</td>
<td>113</td>
</tr>
<tr>
<td>V.</td>
<td>ATTENUATION OF THE FIBROTIC PHENOTYPE OF SCLERODERMA DERMAL FIBROBLASTS BY VIBRATORY STIMULATION: POTENT ANTIFIBROTIC MECHANOTHERAPY</td>
<td>115</td>
</tr>
<tr>
<td>5.1.</td>
<td>Abstract</td>
<td>115</td>
</tr>
<tr>
<td>5.2.</td>
<td>Introduction</td>
<td>116</td>
</tr>
<tr>
<td>5.3.</td>
<td>Materials and Methods</td>
<td>118</td>
</tr>
<tr>
<td>5.4.</td>
<td>Results</td>
<td>124</td>
</tr>
<tr>
<td>5.5.</td>
<td>Discussion</td>
<td>131</td>
</tr>
<tr>
<td>5.6.</td>
<td>Conclusion</td>
<td>137</td>
</tr>
<tr>
<td>VI.</td>
<td>CONCLUSIONS AND FUTURE RECOMMENDATIONS</td>
<td>138</td>
</tr>
<tr>
<td>REFERENCES</td>
<td></td>
<td>143</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Biological pathways significantly enriched in NHDFs subjected to 1X Strain + Vibration relative to 1X Strain</td>
<td>69</td>
</tr>
<tr>
<td>3.2</td>
<td>Biological processes significantly enriched in NHDFs subjected to 1X Strain + Vibration relative to 1X Strain</td>
<td>77</td>
</tr>
<tr>
<td>3.3</td>
<td>Primer sequences used in Real-time RT-PCR</td>
<td>78</td>
</tr>
<tr>
<td>4.1</td>
<td>Variable vibration regimes characterized by varying amplitude, frequency, and duration</td>
<td>99</td>
</tr>
<tr>
<td>4.2</td>
<td>Primer sequences used in Real-time RT-PCR</td>
<td>101</td>
</tr>
<tr>
<td>5.1</td>
<td>Primer sequences used in Real-time RT-PCR</td>
<td>121</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Vibration stimulates differential gene expression including ECM and TGF-β signaling components</td>
<td>76</td>
</tr>
<tr>
<td>3.2</td>
<td>Vibration significantly affects mRNA expression levels of genes associated with matrix remodeling</td>
<td>79</td>
</tr>
<tr>
<td>3.3</td>
<td>Vibration increases expression of profibrotic cytokines, but not fibrous collagens</td>
<td>81</td>
</tr>
<tr>
<td>3.4</td>
<td>Vibration inhibits TGF-β signaling through down-regulation of targets involved in signal reception/transduction and up-regulation of signaling inhibitors</td>
<td>83</td>
</tr>
<tr>
<td>3.5</td>
<td>Densitometric analysis of western blots</td>
<td>84</td>
</tr>
<tr>
<td>3.6</td>
<td>Vibration blocks collagen deposition and mechanical stiffening induced by cyclic strain and exogenous TGF-β1</td>
<td>86</td>
</tr>
<tr>
<td>3.7</td>
<td>Cell number per sponge assessed by DNA content</td>
<td>87</td>
</tr>
<tr>
<td>4.1</td>
<td>Displacement of porous polyurethane substrates in the vibratory bioreactor</td>
<td>100</td>
</tr>
<tr>
<td>4.2</td>
<td>Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either 1X Strain or 1X Strain + Vibration with variable voltage (1.9–4.6 Vrms) and fixed frequency (100 Hz) relative to static control</td>
<td>103</td>
</tr>
<tr>
<td>4.3</td>
<td>Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either 1X Strain or 1X Strain + Vibration with fixed amplitude (approximately 1.28 ± 0.07 mm in amplitude) defined by variable frequency (25–100 Hz) and variable voltage (1.5–3.8 Vrms) relative to static control</td>
<td>104</td>
</tr>
<tr>
<td>4.4</td>
<td>Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either 1X Strain or 1X Strain + Vibration with variable amplitude</td>
<td></td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.64–1.98 mm in amplitude) defined by variable frequency (25–100 Hz) and fixed voltage (3.8 Vrms) relative to static control...</td>
<td>105</td>
</tr>
<tr>
<td>4.5 Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either Static, 1X Strain, or 1X Strain + Vibration with variable duration (2, 4, and 6 hours) at fixed frequency (100 Hz) and fixed voltage (3.8 Vrms) relative to static control</td>
<td>107</td>
</tr>
<tr>
<td>4.6 Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either 1X Strain or 1X Strain + Vibration with variable time cycle (e.g. 2 second on/4 second off in total 6 sec (2s/4s), 4s/2s, and 6s/0s) at fixed frequency (100 Hz) and fixed voltage (3.8 Vrms) relative to static control</td>
<td>108</td>
</tr>
<tr>
<td>4.7 Transient response of vibratory stimulation on the gene expression of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines, but responsive to vibration (6 h/day) for 3 days</td>
<td>110</td>
</tr>
<tr>
<td>5.1 Vibration down-regulates TGF-β signaling on SSc fibroblasts</td>
<td>125</td>
</tr>
<tr>
<td>5.2 Vibration prevents collagen synthesis and accumulation on SSc fibroblasts</td>
<td>126</td>
</tr>
<tr>
<td>5.3 Vibration attenuates the mRNA expression of scleroderma phenotypic markers on SSc fibroblast</td>
<td>128</td>
</tr>
<tr>
<td>5.4 Vibration negatively regulates cyclic strain-induced mRNA expression of scleroderma phenotypic markers on SSc fibroblasts</td>
<td>129</td>
</tr>
<tr>
<td>5.5 Vibration inhibits cyclic strain-induced collagen synthesis and accumulation and mechanical stiffening on SSc fibroblasts</td>
<td>130</td>
</tr>
</tbody>
</table>
CHAPTER ONE
INTRODUCTION

1.1. Extracellular Matrix (ECM)

Extracellular matrix (ECM) is a complex, three-dimensional network of macromolecules that provides structural support and contextual information for cellular growth, communication, survival, adhesion, migration and differentiation [1–3]. It is primarily composed of fibrous collagenous proteins that strengthen the matrix and provide resilience and glycosaminoglycans (GAGs) and proteoglycans that create a highly hydrated gel resistant to compressive forces [4,5]. Cells receive and respond to biochemical and mechanical signals originating from the ECM, and in turn, modulate the ECM through control of matrix assembly. These processes are mediated and regulated through the interaction between cell surface receptors and adhesive glycoproteins in the ECM, such as fibronectin, laminin, vitronectin, and thrombospondins. Each of these glycoproteins has distinct functional domains to bind specific cell surface receptors including integrins, dystroglycans, and syndecans, as well as collagen-binding domains for their incorporation within the matrix network [6,7].

Collagen is the most abundant fibrous protein that constitutes the main structural element of the ECM, provides tensile strength, regulates cell adhesion, supports chemotaxis and migration, and directs tissue development. Of three major collagen types I, II and III, collagen type I constitutes nearly 90% of all the collagen in the human body. Collagens are predominantly produced by fibroblasts that either reside in the stroma or are recruited to it from neighboring tissues. Collagens, like the majority of secreted
proteins, are synthesized in the rough endoplasmic reticulum and undergo extensive co-and post-translational processing. Like all precursor proteins, collagens are initially synthesized as longer precursor proteins called preprocollagens. Following removal of the signal peptide from the preprocollagen precursor, the remaining protein is referred to as a procollagen (or tropocollagen). Procollagen proteins contain globular pro-domains that include an additional 150 amino acids at the N-terminus and 250 at the C-terminus, allowing multiple intrachain disulfide bonds between procollagens and stabilizing procollagen protein and formation of the triple helical structure. Triple helix structures of collagens (collagen fibrils) are composed of two identical alpha chains (e.g. $\alpha 1$) and a different alpha chain (e.g. $\alpha 2$). In addition, collagen proteins have a unique amino acid composition consisting of the repeating sequence, Gly-Pro-X or Gly-X-Hyp (hydroxyproline), where X represents any amino acid except glycine or proline. As much as 35% of a collagen monomer is composed of glycine with another 20-25% being proline. Ascorbic acid is required for the collagen synthesis, especially for the synthesis of hydroxyproline and hydroxylysine [8–10]. Hydroxyproline serves as a stabilizer for the formation of the collagen triple helix structure and hydroxylysine is necessary for formation of intermolecular crosslinks in collagen.

After secretion into the extracellular compartment, collagen fibers are further processed. Proteases remove the globular pro-domains at both the N- and C-termini. The collagen molecules then polymerize to form collagen fibrils. Accompanying fibril formation is the oxidation of certain lysine residues by the extracellular enzyme lysyl oxidase (LOX). Lysyl oxidase (LOX) is an extracellular copper-dependent enzyme that is
also known as protein-lysine 6-oxidase. LOX acts on lysines and hydroxylysines producing aldehyde groups, which will eventually form covalent bonds between tropocollagen molecules, resulting in the formation of highly organized structure with long and thin diameter rod-like protein. By exerting tension on the matrix, fibroblasts are able to organize collagen fibrils into sheets and cables and, thus, can dramatically influence the alignment of collagen fibers.

ECM GAGs are highly anionic polysaccharides secreted into the extracellular matrix independently or covalently bound to core proteins in the form of proteoglycans. Hyaluronic acid (HA) is a high molecular weight GAG that contributes to tissue viscoelasticity. In addition, many GAGs, mostly notably heparin sulfate, electrostatically bind a wide range of basic growth factors, sequestering them within the matrix and creating a reservoir for subsequent release during ECM remodeling and wound healing. Examples include epidermal growth factor (EGF), fibroblast growth factor (FGF) and other signaling molecules such as WNTs [11]. Following release from the ECM, growth factors are localized near cell surface receptors and cell adhesion sites [3,12], regulating ECM architecture and influencing cell behavior controlled by integrin-mediated signaling.

The vast majority of mammalian cells are anchorage-dependent and require adhesion to the ECM matrix for survival and phenotypic function. Cell-ECM adhesion is mediated through transmembrane cell surface receptors that either bind directly to collagen or bind to multi-domain adhesive glycoproteins that contain both collagen-binding (for incorporation into the matrix) and cell-binding domains. Fibronectin is one
of the most important adhesive glycoproteins and plays a central role in mediating mesenchymal cell attachment and migration [2,6,7]. Laminins are a large family of heterotrimeric ECM glycoproteins composed of α, β, and γ chains [7] that play essential roles in the nervous system and epithelial basement membranes.

Integrins are one of the most important and widely studied families of cell adhesion receptors. Integrins are heterodimeric transmembrane receptors formed by non-covalent interactions between α- and β-subunits. At present, 18 α and 8 β subunits have been identified that form 24 functional receptor combinations. Each integrin subunit contains a large extracellular ligand-binding domain, a transmembrane helix, and relatively short (typically 20–70 amino acids) cytoplasmic tail. Integrins bind to extracellular proteins via a small cell-binding recognition sequence, such as the RGD motif (found in proteins such as fibronectin, laminin, or vitronectin). Ligand binding leads to receptor clustering and the recruitment of a variety of cytoplasmic adaptor proteins such as talin, α-actinin, filamin, vinculin and tensin that bind to both the integrin cytoplasmic domain and the actin cytoskeleton [13]. The resulting macromolecular structures, termed focal adhesions, form a physical connection between the ECM and the intracellular actin cytoskeleton. In addition, myosin-mediated contraction further promotes actin coupling and allosteric integrin clustering, as well as the recruitment of other receptors, leading to the formation of signaling complexes for biochemical signal transduction. These structures help transmit mechanical and chemical signals via mechanosensitive components at focal adhesions, dependent upon the mechanical forces they experience as a result of substrate stiffness.
Although integrins and focal adhesions were initially considered to perform solely structural/mechanical functions, it is now recognized that they are critical cell signaling complexes capable of bi-directional signaling. Binding of cytoplasmic proteins to integrin cytoplasmic tails can trigger a change in integrin affinity for extracellular ligand (inside-out signaling/activation). The best example of this is activation of the platelet $\alpha_{\mathrm{IIb}\beta_3}$ integrin, leading to increased fibrinogen binding affinity, in response to intracellular signaling initiated by soluble molecules released in response to vascular injury. Integrins also participate in outside-in signaling initiated by ligand binding. In addition to numerous structural proteins, focal adhesion formation also involves the recruitment of cytoplasmic kinases such as focal adhesion kinase (FAK). FAK-dependent activation of phosphatidylinositol-3’-kinase (PI3K), extracellular signal–regulated kinase (ERK), and c-Jun NH2-terminal kinase, and Akt/protein kinase B (PKB) are important mechanisms by which integrin binding influences cell cycle regulation and protects against apoptosis.

A recently recognized and particularly important characteristic of the ECM is that it is tissue-specific. For example, the ECM in cartilage tissue is enriched in collagen type II and proteoglycans for resisting compressive stress [14], while the glomerular basement membrane (GBM) in the kidney is rich in laminin, collagen type IV, and heparan sulphate proteoglycans that create a selective filtration barrier [15]. Thus, tissue-specific cells in response to biochemical and biomechanical signals in the local microenvironment produce an ECM with distinct biochemical composition and biomechanical properties required to support cellular and tissue function.

1.1.1. ECM Remodeling
Although it was previously believed that the ECM was a static structure that changed only in response to growth or injury, it is now recognized that the ECM experiences complex and dynamic remodeling throughout life. [16]. Thus, precise orchestration of the ECM remodeling process is crucial to the maintenance of normal function [17,18]. Careful balance between matrix synthesis and enzymatic degradation regulated by growth factors, cytokines and mechanical stimulation is important for ECM remodeling. Specifically, ECM degradation is controlled by various enzymes, including heparanase, cathepsins, hyaluronidases, matriptases, various serine and threonine proteases [19], the large superfamily of metzincins, which includes ADAMs (a disintegrin and metalloproteinases), ADAMTSs (ADAMs with thrombospondin motifs), and matrix metalloproteases (MMPs) [20,21]. These enzymes regulate matrix components of the basement membrane as well as proteins and proteoglycans of connective tissues. As noted above, ECM degradation also liberates sequestered growth factors, providing a mechanism for coupling their activity to matrix turnover [22].

1.1.2. Regulation of Matrix Remodeling

1.1.2.1. Growth Factors and Cytokines

ECM remodeling is controlled by wide variety of different growth factors and cytokines, including TGF-β, PDGF, bFGF, EGF, VEGF, TNFα and interleukins (IL) [23]. TGF-βs have a wide spectrum of activities, regulating cell proliferation and differentiation as well as synthesis of many ECM components. TGF-β1 induces monocytes and macrophages to synthesize a number of cytokines such as IL-1β, TNF-α and TGF-β1 itself [25,26]. These cytokines affect both fibroblasts and inflammatory cells
to promote the production of new matrix proteins such as collagens I, III, and V, fibronectin, proteoglycans and other ECM components [40]. In addition, PDGF induces fibroblast and smooth muscle cell migration and proliferation and stimulates the activation of macrophages [48-53], resulting in increased procollagen synthesis, collagenase activation and fibronectin gene expression [54-56]. Fibroblast Growth Factors (FGFs) are key growth factors involved in angiogenesis, directing endothelial cell migration, proliferation, and plasminogen activator synthesis [52,64-65]. FGFs have been shown to accelerate granulation tissue formation, increase fibroblast proliferation and collagen accumulation, and enhance vascularization [69].

Although many cytokines play important roles in matrix remodeling, TGF-β1 has clearly been identified as the prototypical ‘profibrotic’ cytokine. Accumulated evidence has demonstrated that TGF-β1 promotes myofibroblast differentiation and increases collagen synthesis and accumulation that ultimately leads to fibrosis of many organs [24–26]. TGF-β is also known to negatively regulate the production of MMP1 and MMP7 [27,28], but induce TIMP1 expression [29]. As noted above, the matrix metalloprotease (MMP) family plays a pivotal in the ECM remodeling, collagenous proteins in particular. Specifically, the down-regulation of MMP1 (a.k.a collagenase-1) is involved in fibrotic diseases characterized by exaggerated ECM synthesis [27,28,30]. Increased levels of TIMP-1 as an inhibitor of many active MMPs has also been observed in fibrotic diseases [27,29]. In addition to its direct profibrotic activity, TGF-β1 also indirectly promotes fibrosis through the downstream cytokines CTGF and EDN1. Numerous studies have shown that TGF-β1 is a potent inducer of CTGF expression [31] and CTGF functions as
a downstream mediator of specific TGF-β1 actions on connective tissue cells, where it stimulates cell proliferation and ECM synthesis. CTGF has been implicated as a key regulatory target in complex biological and pathological processes particularly involving connective tissue formation in wound repair or fibrotic disorders [32,33]. Endothelin 1 (EDN1) is also a downstream mediator of the profibrotic action of TGF-β in the context of fibrotic diseases; increased EDN1 expression by fibroblasts is a hallmark of fibrotic disease and it is known to act synergistically with TGF-β [34,35].

1.1.2.2. Mechanical Microenvironment

All cells in multicellular organisms are exposed to mechanical forces of varying magnitude that play an integral role in regulating cell behaviors such as spreading, migration, proliferation and differentiation. Cells sense the mechanical microenvironment via transmembrane molecules and exhibit different mechano-responsive behaviors, depending upon the mechanical loading types and properties of the ECM. One important characteristic of the mechanical microenvironment that cells experience is matrix stiffness, which ranges from 50 Pa (e.g. blood fluid or mucus), 1~2 kPa (e.g. brain), 2-5 kPa (e.g. lung), 5-6 kPa (e.g. skin), 12~13 kPa (e.g. smooth muscle), 1~3 MPa (e.g. cartilage) and up to 1~20 GPa (e.g. bone) [16][36]. As described earlier, cells detect the stiffness of the microenvironment by pulling on the ECM and such processes are dependent on ECM adhesions that act as a bridge to transmit force between the ECM and the cellular cytoskeleton, in which myosin-based contractility acts as a primary regulator of cellular contractile forces.
While TGF-β increases matrix deposition and stiffness, matrix stiffness is also crucial to promote the expression of various ECM proteins and has been widely studied using collagen constructs with defined matrix stiffness [37–40]. Elevated expression of collagen type 1, 2, 3 and 6, fibronectin and α-actin were observed and fibroblasts developed enlarged actin microfilament bundles and organized fibronectin into extracellular fibrils during culture in stressed collagen gels [41,42]. Interestingly, the inhibitors of matrix metalloproteinases such as TIMP1 and TIMP3 were significantly induced in response to increased substrate stiffness [38]. Meanwhile, fibroblasts cultured in freely floating, relaxed collagen lattices exhibited decreased collagen synthesis [43], but increased synthesis and activation of matrix metalloproteinase (MMP)-1 [44][45]. These studies demonstrate that matrix rigidity affects not only the synthesis and production of ECM components but also other targets associated with fibrotic diseases including the deposition and organization of these components.

A second important characteristic of the mechanical microenvironment that influences matrix synthesis is the exposure of cells to applied external loads/forces. For instance, fibroblasts in ligament, chondrocytes in cartilage, and endothelial cells (EC) in vascular tissues experience tension, compression and shear stress, respectively, regulating their matrix transcription and production [50–53]. Mechanical stretch plays an active role in many physiological processes such as muscle contraction. It has been demonstrated that the stretching forces stimulated cells in many engineered tissues (e.g. muscle, ligament, tendon and vasculature) to enhance their organization, strength and functionality [50, 54]. Smooth muscle cells (SMCs) have shown directed cell alignment
and migration in collagen substrates parallel to the direction of mechanical stretch [55], down-regulated expression of bone-associated genes, and decreased deposition of calcium [56]. Cyclic stretch promoted myocardial cells to form interconnected and longitudinally oriented cardiac muscle bundles [57]. Additionally, cardiac cells in fibrin and fibroblasts in collagen tend to increase collagen synthesis and deposition under cyclic stretch [58, 59]. Compressive and shear stresses selectively affect certain types of cells including chondrocytes, mesenchymal stem cells (MSCs) and endothelial cells (ECs). Dynamic compression greatly increased equilibrium modulus, glycosaminoglycan and hydroxyproline content of chondrocytes in an agarose hydrogel construct [60]. Dynamic compression also enhanced production of cartilage matrix by MSCs cultured in HA hydrogels [53]. ECs in the human vascular system experience shear stress from the passage of blood fluid through the cardiovascular system, which plays an important role in regulating vascular matrix composition and function [64, 65]. Collectively, exposure to physiologically relevant external mechanical stimulation activates signaling pathways to stimulate tissue-specific matrix expression.

1.2. Wound Healing

The wound healing process is a dynamic and highly regulated process of cellular, humoral and molecular events involving cell migration, proliferation, and matrix remodeling [46,47]. Following tissue injury, the first stage of acute wound healing is dedicated to hemostasis and the formation of a provisional wound matrix, and the inflammatory process, which is divided into an early phase with neutrophil recruitment and a late phase with the appearance and transformation of monocytes. The proliferative
phase involves re-epithelialization of the wound surface, formation of granulation tissue 
and the restoration of the vascular network through neovascularization and angiogenesis.
The formation of granulation tissue stops through apoptosis of the cells, then the newly 
synthesized ECM components undergo remodeling resulting in the formation of a mature 
scar, which is the physiological endpoint of wound repair and directly linked to the extent 
of the inflammatory process throughout wound healing.

1.2.1. Hemostasis

The initial injury results in an outflow of blood and lymphatic fluid. Immediately 
after injury, blood vessels constrict and the process of initial reparative coagulation is 
initiated [48]. Minor injury to the endothelial layer exposes a number of matrix proteins 
including type IV collagen, laminin, and Von Willebrand factor (VWF), and in some 
situations, fibronectin, whereas more severe injuries exposing the smooth muscle layer or 
interstitial ECM will expose fibrillar collagens, elastin, microfibrils, and other ECM 
proteins [49]. Although platelets can bind directly to these ECM proteins, interactions 
with VWF and fibrinogen are most important for hemostasis [50]. Circulating VWF can 
be bound directly by platelets or can bind to exposed collagen fibers. Especially when 
activated by collagen, GPVI, a receptor for fibrillar collagens provides stimulatory 
signaling that triggers various platelet responses such as activation of $\alpha_{\text{IIb}\beta_3}$ integrin, 
granule release, and cytoskeletal rearrangements. Platelets bind and aggregate together 
through two VWF binding receptors GPIb-V-IX complex and the platelet-specific 
integrin, $\alpha_{\text{IIb}\beta_3}$ that bind to the binding site, KQGADV sequence near the carboxyl 
terminus of the $\gamma$ chain of fibrinogen [49]. Once the platelets are activated and engaged
by ligand, GPIb-V-IX and by soluble by-products of the clotting cascade including thrombin that cleaves circulating fibrinogen to fibrin and activates Factor XIIIa, causes “inside-out” activation of $\alpha_{IIb}\beta_3$. Subsequently, the interaction with other ECM ligands deposited on the surface of the thrombus further enhances platelet aggregation and formation of an occlusive thrombus, contributing to the mechanical stability of the fibrin network and also undergoing degranulation to release cytokines and growth factors. After an initial period of vasoconstriction, vasoactive amines released from the platelets and mast cells cause surrounding vessels to become leaky, thus allowing a massive influx of inflammatory cells and other blood cells required into the wound area. The final product of the coagulation process, the fibrin clot, is not only important for hemostasis, but also provides a provisional matrix for cell migration in the subsequent phases of the inflammatory and proliferative phases.

1.2.2. Inflammatory Phase

As described earlier in the introduction, the inflammatory phase of the wound healing cascade is initiated and activated during the hemostasis and coagulation phase. Overall inflammatory events are roughly divided into an early phase with neutrophil recruitment and a late phase with the appearance of monocytes and their transformation into tissue macrophages. Due to the response of the activated complement pathway, degranulated platelets, and by-products of bacterial degradation, neutrophils are recruited to the injury site through chemotaxis. They perform phagocytosis and secrete high levels of proteases to kill local bacteria and degrade necrotic tissue [51]. Neutrophils also secrete pro-inflammatory cytokines and chemokines that are responsible for the
recruitment and activation of additional polymorphonuclear leucocytes, macrophages, mast cells, and T cells Th1 and Th2 lymphocytes that are involved in the inflammatory phase through the release of mediators such as tumor necrosis factor (TNF)-α, IL-1β and IL-4, IL-5, IL-6, IL-8, IL-10, and growth factors including transforming growth factor (TGF)-α, TGF-β, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF).

Macrophages play an integral role in not only supporting the clearance process by performing phagocytosis of pathogens and cell debris [52,53], but also initiating the proliferative phase through the synthesis of numerous mediators and growth factors that promote cell proliferation and the synthesis of ECM molecules by resident cells [54]. Although the resting, non-activated macrophages produce only low levels of pro-inflammatory mediators, once exposed to pro-inflammatory cytokines including interferons, lipopolysaccharide (LPS) or other microbial products (such as bacterial-derived unmethylated CpG-DNA), or damage-associated molecular patterns (DAMPs; such as heat-shock proteins, high mobility group box proteins (HMGB1), and molecular fragments of the extracellular matrix that are generated following tissue injury), macrophages develop a pro-inflammatory, classically activated phenotype (M1). Following activation, macrophages themselves produce a large number of mediators and cytokines including interleukin-1, interleukin-6, interleukin-12, TNFα, and inducible nitric oxide synthase (iNOS) [26,55]. Recent studies of macrophages derived from skin wounds as well as sponges implanted subcutaneously in mice demonstrate that macrophages exhibit multiple phenotypes that change during the inflammatory phase of
the wound healing process [56]. These studies suggest that M1-like macrophages are common in the early phases of repair, while alternatively-activated (M2) macrophages, with less pro-inflammatory cytokines and elevated markers of alternative activation, including CD206 and arginase 1 (Arg1), are common in later stages of repair [57–59]. Thus, the transition from a pro-inflammatory to a pro-healing phenotype in macrophages is an essential process in normal wound healing. In addition, T lymphocyte that appear in the wound bed in the late inflammatory phase also regulate the phenotype shifts from pro-inflammatory macrophages to macrophages with anti-inflammatory/proangiogenic cytokine activities, suppressing inflammatory responses and inducing neovascularisation and fibroblast and epithelial cell proliferation, thereby leading to the transition from inflammation to repair [53]. The inflammatory response to injury is therefore essential for supplying growth factors and cytokine signals that are important for cell recruitment and activation, which are also crucial for the subsequent repair mechanisms in adult mammals [51,60].

1.2.3. Proliferative Phase

The main characteristics of the proliferative phase are re-epithelialization, replacement of the provisional matrix with newly formed granulation tissue, and restoration of the vascular network [61,62]. A variety of cytokines and growth factors released by inflammatory cells stimulate cells such as fibroblasts, endothelial cells, and keratinocytes to promote cellular recruitment and proliferation, produce ECM proteins and glycoproteins, and initiate angiogenesis. Fibroblasts secrete bFGF, TGF-β, PDGF, insulin-like growth factor 1 (IGF-1), and keratinocyte growth factor (KGF). Endothelial
cells produce vascular endothelial growth factor (VEGF), bFGF, and PDGF. Keratinocytes also synthesize TGF-β, TGF-α, and KGF. VEGF and FGF2 stimulate blood vessel formation and re-epithelialization. PDGF and TGF-β play a particularly important role in stimulating fibroblast proliferation and migration into the provisional matrix.

Under the control of regulating cytokines like IFN-γ and TGF-β, fibroblasts initially synthesize a matrix rich in glycosaminoglycans (GAGs) and glycoproteins including fibronectin and tenasin [61–63] that serves as a transitional matrix to support subsequent collagen deposition and assembly. It is characterized by a high density of fibroblasts, granulocytes, macrophages, capillaries and loosely organized collagen fibrils. It serves to close tissue gaps and provide temporary mechanical stabilization of the wound. Growth factors released by macrophages and other cells that are recruited into the wound area further facilitate the migration of keratinocytes, fibroblasts and endothelial cells and the synthesis of new collagens, the subtypes I and III. Early in normal wound healing, type III collagen predominates but is later replaced by type I collagen. The fibril assembly, organization, and degradation are mediated by the binding of small leucine-rich proteoglycans (SLRPs) to different types of collagens through the covalent links of glycosaminoglycan (GAG) side chains [64,65]. The interaction of SLRPs with collagens has been shown to enhance fibril stability and to protect fibrils from proteolytic cleavage by various collagenases [66]. Especially, decorin is known to associate with collagen fibrils by binding to collagen type I, II, III, IV, VI, and XIV in all connective tissues. The
GAG chain of decorin also binds tenascin-X and mediates its interaction with collagen fibrils, thereby contributing to ECM integrity [67].

Angiogenesis is a complex cascade of molecular events in the wound site to restore vascular circulation and deliver oxygen and nutrients to support tissue repair [68,69]. The process is initiated by growth factors, such as VEGF, PDGF, bFGF and the serine protease thrombin. The first step in new vessel formation is the binding of growth factors to their receptors on the endothelial cells of existing vessels, thereby activating intracellular signaling cascades. The activated endothelial cells secrete proteolytic enzymes that degrade the basal lamina by secretion and activation of MMPs. Endothelial cells then migrate and proliferate into the provisional wound matrix, a process known as ‘sprouting’ [70]. The sprouts form small tubular canals that interconnect to others forming a neovessel loop. Thereafter, the new vessels differentiate into arteries and venules and mature by further stabilization of their vessel wall via the recruitment of pericytes and smooth muscle cells.

Finally, the synthesis of collagen increases throughout the wound while adjusting a balance between synthesis and degradation of the ECM [63], and the number of maturing fibroblasts is subsequently reduced by myofibroblast differentiation, down-regulated along with decreased vascularity, and terminated by c-Myc-induced cell apoptosis [71–73]. Several in vitro studies suggest that fibroblast apoptosis is mediated by the cell surface interactions of Fas (CD95) with its ligand, FasL (CD95L) on the cell membrane. Fas is a cell surface transmembrane receptor of the tumor necrosis factor receptor family that activates an intrinsic apoptotic suicide program in cells upon binding
its ligand FasL. Fas/FasL binding initiates the apoptotic signaling pathway through c-Myc that ultimately leads to the activation of caspase proteases and the apoptosis of myofibroblasts, restoring cell density to pre-injury levels [74,75].

1.2.4. Remodeling Phase

As the last phase of wound healing, the remodeling phase is responsible for the development of new epithelium and final scar tissue formation. Since 1971, myofibroblasts, after discovery by Gabbiani et al. in the granulation tissue, have been identified to play an important role in wound contraction and scar formation during the wound healing process [76,77]. During the remodeling phase, myofibroblasts are responsible for massive synthesis and production of ECM proteins, including collagen types I-VI and XVIII, glycoproteins, and proteoglycans and other matrix molecules such as laminin, thrombospondin, glycosaminoglycans (GAGs), hyaluronic acid (HA), and heparan sulfuate (HS), as well as matrix-modifying proteins such as matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs).

Meanwhile, the components of the wound ECM undergo certain changes; collagen type III, the major component of the granulation tissue, is slowly degraded and replaced with collagen type I. The collagen fibres are reorganized in an equilibrium-producing fashion and are stabilized by increased covalent cross-linking of collagen molecules by the enzyme lysyl oxidase (LOX), which is secreted into the ECM by fibroblasts [52]. Eventually, the initially disorganized collagen bundles are decreased, the newly oriented and cross-linked collagen matrices are increased over time.
In addition to producing matrix components, myofibroblasts also generate contractile forces that ultimately lead to re-organization of collagen matrix and wound closure [78]. Initially, cell traction forces (CTFs) generated by fibroblasts lead to the migration of more fibroblasts from the surrounding dermis and subcutaneous tissues into the wound [79]. As contraction proceeds and resistance increases, fibroblasts are differentiated into myofibroblasts that express the contractile proteins, α-smooth muscle actin (α-SMA) [80]. The bundles of cytoplasmic microfilaments are associated with contractile proteins including non-muscle myosin that provide large contraction forces and lead to re-organization of collagen matrix [76,81,82].

The maturation of granulation tissue also involves a reduction in the number of capillaries as small vessels are aggregated into larger ones and collagens further accumulate, accompanied by a decrease in the amount of GAGs and proteoglycans. Moreover, as new blood vessels enter the wound repair area and the oxygen tension returns to a normal level, oxygen binds to hypoxia-inducible factor (HIF), a highly conserved transcription factor that controls the expression of numerous angiogenic factors, and blocks its activity leading to a decreased synthesis of VEGF [69,83]. Furthermore, as the angiogenic process diminishes, the wound blood flow declines and the wound metabolic activity slowly returns to homeostatic levels.

1.3. Fibrosis

Fibrosis describes a complex, diverse group of chronic pathological diseases [84] characterized by the excessive accumulation of fibrous connective tissue such as collagens and fibronectins and eventually results in permanent scarring, ultimately organ
malfunction and death [16,85,86]. Fibrosis affects nearly every tissue in the body including the lung, skin, heart, kidney and it is estimated that 45% of deaths in the United States can now be attributed to some type of chronic fibrotic disease [87]. There are many triggers that can initiate the progressive fibrotic disease. Examples include inherited genetic disorders, persistent infections, recurrent exposure to toxins, irritants or smoke, chronic autoimmune inflammation, minor human leukocyte antigen mismatches in transplants, myocardial infarction, high serum cholesterol, obesity, and poorly controlled diabetes and hypertension. Regardless of all of these events, a common feature in all fibrotic disorders is the activation of fibroblast to myofibroblasts, which is the key mechanism in fibrotic pathology.

Myofibroblasts are rarely found in healthy human physiology, however they become vastly increased after injury and play a critical role in the wound healing response. The myofibroblast phenotype is defined by the formation of a contractile apparatus with associated contractile proteins, such as α-smooth muscle actin (α-SMA) and non-muscle myosin, increased levels of ECM synthesis and secretion, and resistance to apoptosis [40,88–91]. Myofibroblasts are also well-characterized to differentiate from resident fibroblasts in vivo and in vitro in response to profibrotic cytokine stimulation. Many cytokines released by inflammatory cells such as TGF-β1, interleukin (IL)-1β, IL-6, IL-13, IL-33, as well as prostaglandins and leukotrienes facilitate the activation of fibroblasts into myofibroblasts. In turn, fibroblasts produce and secrete cytokines such as TGFβ1, IL-1β, IL-33, CXC, and CC chemokines, allowing them to assist in the activation and migration of resident immune cells such as macrophages [51]. Moreover,
myofibroblasts have been observed to differentiate from various other precursor cells, including epithelial cells, endothelial cells, pericytes, multipotent monocytes, and fibrocytes. However, how they lead to differential fibrotic responses in different tissues remains to be determined [92].

Heart failure, the clinical manifestation of numerous forms of cardiovascular disease (CVD), is a devastating disorder that causes substantial mortality in the United States, accounting for nearly 600,000 deaths per year. Nearly all etiologies of heart disease involve pathological myocardial remodeling characterized by excessive deposition of ECM proteins by cardiac fibroblasts, which reduces tissue compliance and accelerates the progression to heart failure [93]. Unlike other organs, the heart has a limited regenerative capacity after injury, and instead, repair processes involve the removal of necrotic cardiomyocytes followed by fibrotic scar tissue replacement that acts to preserve myocardial structural and functional integrity. Cardiac fibroblasts play an essential role in cardiac wound healing processes consisting of inflammation, proliferation of non-myocytes, and scar maturation [94]. After an acute myocardial injury, various pro-inflammatory cytokines and profibrotic factors are upregulated that lead to increased proliferation of cardiac fibroblasts and ultimately, the phenotypic transition to the myofibroblasts. During this maturation phase, myofibroblasts produce elevated amount of collagens and other ECM proteins in order to maintain the structural integrity and pressure-generating capacity of the heart, because a loss of integrity in the mechanical strength of the ventricle may lead to myocardial dysfunction. In the advanced phases of fibrotic scar formation, activated myofibroblasts express contractile proteins
such as α-SMA, resulting in elevated stiffness of the developing scar tissue. These processes eventually contribute to pathological cardiac remodeling, leading to altered ventricular structure and compliance, and a concurrent progression into heart failure [95].

Chronic kidney disease (CKD), an epidemic affecting 10-13% of all adults worldwide and accounting for about a million deaths per year [96], results in the widespread tissue scarring leading to complete destruction of kidney parenchyma and end-stage renal failure (ESRD). Renal fibrosis, the final common manifestation of a wide variety of CKDs is characterized by glomerulosclerosis, tubulo-interstitial fibrosis, inflammatory infiltration, and loss of renal parenchyma characterized by tubular atrophy, capillary loss, and podocyte depletion [97] and gradually develops in response to prolonged hypertension and diabetic glucose dysregulation [86,98]. Like other fibrotic diseases, the pathogenesis of renal fibrosis is characterized by an excessive accumulation and deposition of ECM [99–101]. After the initial injury, the affected kidney tissues undergo a series of events in an attempt to repair and recover from the damage. Kidney resident cells are activated to produce pro-inflammatory cytokines, resulting in the infiltration of inflammatory monocytes/macrophages and T cells to the injured sites. Depending upon the nature and sites of injury, glomerular or interstitial infiltrating inflammatory cells become activated, and produce injurious molecules such as reactive oxygen species, as well as fibrogenic and inflammatory cytokines. These mediators stimulate glomerular mesangial cells, fibroblasts, and tubular epithelial cells to undergo myofibroblastic activation or transition and produce a large amount of ECM components. Continuous, dysregulated deposition of ECM proteins such as type I and type III collagen
and fibronectin results in fibrous scars and distorts the fine architecture of kidney tissues, leading to the collapse of renal parenchyma and the loss of kidney function.

Liver fibrosis and its end-stage, cirrhosis, remain a massive health care burden worldwide [102] . The main causes of liver fibrosis are chronic viral hepatitis B or C infection, autoimmune and biliary diseases, alcoholic steatohepatitis (ASH) and, increasingly, nonalcoholic steatohepatitis (NASH), that lead to the replacement of functional parenchyma with scar tissue and ultimately, severe architectural and vascular distortion in liver tissue [102]. In addition, the clinical complications of cirrhosis involves ascites, renal failure, hepatic encephalopathy, and variceal bleeding and liver transplantation is indicated as the only effective therapy [103]. Based on the central etiology of fibrogenesis, the activation of tissue fibroblasts into ECM producing myofibroblasts is also considered as a primary mediator in liver fibrosis. Within the liver, hepatic stellate cells (HSC) that reside in the subendothelial space of Disse, which contains a low-density basal membrane–like matrix that is essential for maintaining the differentiated function of parenchymal cells, are the main effector cells of fibrosis [104,105]. Quiescent HSCs become activated to a contractile myofibroblast-like matrix-secreting phenotype, and secrete fibrillar collagens. In addition, the altered balance between matrix synthesis and degradation e.g. increased expression of tissue inhibitors of metalloproteinases (TIMP) and decreased matrix-degrading MMP activity further leads to progressive deposition of ECM matrix in the space of Disse [106], resulting in the loss of the normal fenestrations of the endothelial lining, creating impaired metabolic
exchange between portal venous flow and hepatocytes, eventually resulting in liver failure [107].

Idiopathic pulmonary fibrosis (IPF) is the most progressive and lethal form of pulmonary fibrotic diseases, with no proven effective therapy, and with lung transplantation remaining the only viable intervention in end-stage disease [108,109]. Although the precise mechanisms that drive the development of pulmonary fibrotic disease remain incompletely understood, aberrant lung fibroblasts, loss of alveolar epithelial cells, and excessive accumulation of ECM appeared to be responsible for pulmonary fibrosis [110]. The continuous activation of lung fibroblasts to a synthetic and contractile myofibroblast phenotype leads to the excessive ECM deposition and contraction of ECM matrix [111,112]. In addition, several lines of evidence demonstrate that the loss of normal alveolar epithelial cells that is important for gas exchange and production of alveolar ECM components and replacement by hyperplastic type II cells or bronchiolar cuboidal cells contribute to the perpetuation of the fibrotic scarring [113]. Thus, altered lung mesenchymal cells coupled with alveolar epithelial cell injury result in the destruction of pulmonary architecture.

Hypertrophic scars and keloids result from abnormal wound healing in response to skin injuries such as surgery, burns, trauma, and inflammation in predisposed individuals [114,115]. Hypertrophic scars usually occur shortly after injury and may subside with time, however, leaving behind an unsightly wide gap of thinned dermis in the wound edges. On the other hand, keloids may infiltrate into surrounding normal tissue, and manifest over time without a quiescent or regressive phase. Hypertrophic and
keloid scars begin as the result of prolonged inflammatory and proliferative responses particularly in the injury to deep dermis. Hypertrophic scar fibroblasts respond normally to growth factors and demonstrate only a modest increase in collagen production. Fibroblasts in keloids, however, have different properties than those seen in normal skin and hypertrophic scars [116–118]. Fibroblasts from keloid scars respond abnormally to stimulation, show a greater capacity to proliferate and produce high levels of collagen type I, elastin, fibronectin, and proteoglycan. For example, collagen synthesis is approximately 20 times as great as that in normal unscarred skin and three times as great as in hypertrophic scars. Keloid tumors then grow to reach a certain size and may remain that size indefinitely. Aside from high collagen synthesis and proliferation of fibroblasts in keloids, keloid-derived fibroblasts also show a high rate of fibronectin biosynthesis that is as much as four times as high as those of fibroblasts derived from normal scars and normal dermis. With regards to increased expression of TGF-β and its stimulation of fibroblast proliferation and migration in wounds, in keloidal tissue, TGF-β is overproduced and poorly regulated through normal autocrine signaling mechanisms [118]. Moreover, keloid fibroblasts have greater numbers of growth factor receptors and respond more intensely to growth factors such as TGF-β and PDGF. Decreased synthesis of molecules that promote matrix breakdown (e.g., MMPs) and disturbed apoptosis mechanisms have also been reported in hypertrophic scars and keloids, leading to uncontrolled, progressive collagen synthesis in wounds. Furthermore, several studies also indicate that both types of fibrotic scarring can develop from extremely increased mechanical skin tensions induced by high collagen synthesis and proliferation of
fibroblasts [115]. Specifically, scars located in certain areas of the body (e.g., sternum, deltoid, and upper back) frequently become hypertrophic.

Fibrosis is also a major pathological feature of many chronic autoimmune diseases. For example, scleroderma (systemic sclerosis, SSc) is a connective tissue disease defined by autoimmunity and inflammation, progressive tissue fibrosis and widespread vascular disorder [119], affecting over 100,000 individuals in the United States. Vasculopathy in the larger vessels can manifest as pulmonary arterial hypertension (PAH) or scleroderma renal crisis (SRC), involving fibrointimal proliferation of small vessels and vasospastic episodes triggered by cold or stress (clinically referred to as Raynaud’s phenomenon) and ultimately leads to tissue ischemia. Moreover, dysregulation of the immune system activates autoantibody production, aberrant cytokine and chemokine release, and destruction of the innate immune system, further leading to the activation of fibroblasts and recruitment of progenitor and/or stem cells from the bone marrow and circulation. Prolonged inflammation and the presence of local and systemic profibrotic factors facilitate the transdifferentiation of resident fibroblasts and recruited cells such as pericytes, fibrocytes, endothelial and endothelial progenitor cells into myofibroblasts, which are responsible for the deposition of large quantities of ECM components [120]. Progressive replacement of tissue architecture by ECM components such as collagen and fibronectin results in functional impairment of affected organs. Ulcerative colitis (UC) and Crohn's disease (CD) are intestinal fibrosis resulting from chronic inflammation and impairment of intestinal wound healing. In UC, the involvement of the mucosal and submucosal layers causes a thickening of the
muscularis mucosae with accumulation of ECM that may contribute to shortening or stiffening of the colon, whereas in CD, the transmural nature of the inflammatory process is followed by bowel wall thickening, and eventually formation of stricture and stenosis [121]. In addition, myelofibrosis (MF) is characterized by a clonal haemopoietic stem cell proliferation associated with a characteristic stromal pattern, a leuko-erythroblastic blood film and elevated levels of various inflammatory and pro-angiogenic cytokines [122].

1.3.1. TGF-β Signaling and Fibrotic Diseases

As the key molecules in the activation and progression of fibrotic disorders, the profibrotic cytokines, transforming growth factor (TGF)-β and connective tissue growth factor (CTGF) are considered as critical mediators in the fibrotic program, stimulating massive ECM synthesis and deposition by local fibroblasts/myofibroblasts [24,123–126]. The imbalance between enhanced production and deposition and impaired degradation of ECM components, primarily collagens results in tissue fibrosis.

TGF-β1 is implicated in a wide range of cell functions, critically regulating tissue homeostasis and repair, immune and inflammatory responses, ECM deposition, cell differentiation, and growth [127,128]. In mammals, three structurally similar isoforms of TGF-β, TGF-β1, 2, and 3 are expressed in a distinct tissue specific manner under control of different promoters [129,130]. Although the in vitro functions of the three isoforms are similar, however their in vivo effects are distinct. Loss-of-function experiments in mice have demonstrated that each TGF-β isoform plays an independent role in embryonic development highlighting their non-compensated functions. Moreover, although all three
isoforms are expressed in fibrotic tissues, the development of tissue fibrosis is primarily attributed to TGF-β1 [131].

Members of the TGF-β superfamily elicit signaling through distinct combinations of transmembrane type I (TGFβRI) and type II receptors (TGFβRII) [132]. Type I and type II receptors are serine/threonine kinases that form a heteromeric complex. In response to ligand binding to type II receptor, a stable complex with type I receptor is formed allowing its transphosphorylation and thus activation of type I receptor kinases. Among the seven known mammalian type I receptors termed activin receptor-like kinase (ALK1-7), ALK5 is expressed on many different cell types and is utilized by TGF-β1 for signaling [133]. There are additional receptors for TGF-β, including the type III receptors β-glycan and endoglin that serve as accessory co-receptors and facilitate TGF-β ligand binding to the type II receptor [134].

The signaling pathway for TGF-β further propagates the signal through phosphorylation of the Smad proteins [135]. The eight mammalian Smads can be grouped into three functional classes: the receptor-regulated Smads (R-Smads, Smadl, Smad2, Smad3, Smad5, and Smad8), the common mediator Smad (Co-Smad, Smad4), and the inhibitory Smads (I-Smads, Smad6 and Smad7). The R-Smads, Smad2 and Smad3 are phosphorylated directly by the TGF-β receptor (ALK5) [136]. Subsequently, the R-Smads form complexes with the Co-Smad, Smad4, and translocate to the nucleus, where they activate or repress gene transcription depending on their recruitment into transcriptional complexes of co-activators such as p300, CBP, AP-1, signal protein 1 (SPI) or co-repressors such as c-Ski, SnoN, transforming growth inhibiting factor, or
Smad nuclear-interacting protein-1 (SNIP1) [137]. The I-Smads serve as negative regulators: Smad6 and Smad7 antagonize TGF-β signaling by binding to type I receptor (Smad7) or by competing with activated R-Smads for binding to Co-Smad4 (Smad6) [138]. Moreover, inhibitory Smads (I-Smads) recruit the E3 ubiquitin-protein ligases Smurf1 and Smurf2 that target Smad proteins for proteasomal degradation, thereby terminating Smad-mediated signaling [139,140]. Smad7 expression is also induced by Smad3 [141], therefore providing auto-inhibitory feedback loop that suppresses TGF-β-mediated effects [142].

TGF-βs and their receptors are consistently upregulated and activated in fibrotic diseases and modulate fibroblast phenotype and function [24,126,143]. Relevant to fibrosis, TGF-β is rapidly induced, assisting in recruiting neutrophils, macrophages, and fibroblasts, which in turn release more TGF-β and the elevated expression of TGF-β receptors results in further activation of receptor-mediated TGF-β signaling transduction (72, 73). In dermal fibrotic lesions of scleroderma patients, elevated TGF-β levels are observed at the lesion of scar tissues (74). Furthermore, TGF-β1 stimulation leads to activation and differentiation of fibroblasts to myofibroblasts, the key effector cells in fibrotic states [121,143].

In addition, TGF-β signaling has been shown to play an important role in epithelial-to-mesenchymal transition (EMT) [133,144,145]. While EMT is a normal physiological process necessary for proper tissue development, the pathologic induction of EMT is associated with fibrotic diseases [146,147]. TGF-β induces the mesenchymal transdifferentiation of epithelial cells, resulting in the increased expression of genes
associated with myofibroblast phenotype, the disruption of cell–cell and cell–matrix interactions, degradation of the surrounding ECM, and actin reorganization.

In normal wound healing, myofibroblasts are required for tissue repair; however, in pathologic conditions, activated myofibroblasts become the critical effectors of fibrotic disorders. To repair, regenerate and restore homeostasis after injury, tissue-resident fibroblasts are activated and transform into myofibroblasts, contractile cells expressing α-SMA, desmin, and myosin bundles. It is well established that the first changes that fibroblasts undergo during myofibroblastic modulation are the acquisition of contractile stress fibers composed of cytoplasmic actins and the production of cellular fibronectin [71]. Under mechanical stress, fibroblasts will differentiate into proto-myofibroblasts, which form cytoplasmic actin-containing stress fibres that terminate in fibronexus adhesion complexes [148]. Myofibroblasts sustain the contraction by using the specialized adhesion complex that allows intracellular actin filaments to bind with extracellular fibronectin domains, ED-A fibronectin expressed and organized by proto-myofibroblasts [78]. As noted above, in the presence of mechanical stress along with the presence of TGF-β, the differentiation of proto-myofibroblasts into myofibroblasts leads to more extensively developed stress fibres and large fibronexus adhesion complexes. Subsequently, this contraction is stabilized and replaced by the deposition of ECM, collagen in particular, leading to maturation of the ECM microenvironment. During normal wound healing, myofibroblasts undergo apoptosis after re-epithelialization of the wound. However, in pathological wound healing and fibrotic diseases, the myofibroblasts became resistant to programmed cell death, persist in the wound site and lead to tissue
deformation. The long-term existence and uninterrupted matrix accumulation by myofibroblasts leads to destruction of normal ECM structure and its replacement with fibrous tissues, which is particularly evident in many fibrotic events: hypertrophic scars developing after burn injury and in the fibrotic phase of scleroderma [84,149].

1.3.2. Reversibility of Fibrosis

It is generally considered that once fibrosis has begun, it cannot be reversed due to the activated myofibroblasts, uncontrolled collagen synthesis, and LOX-mediated crosslinking of collagen matrices. As described earlier, fibrotic processes are characterized by an excessive accumulation of collagen with increased levels of hydroxyallysine-derived cross-links. The occurrence of these cross-links appears to be an important criterion in assessing the reversibility of fibrosis. In normal skin, levels of pyridinolines derived from hydroxyallysine aldehyde is very low, however, during the fibrotic process, increased formation of pyridinoline cross-links is observed [150,151]. Elevated hydroxyallysine cross-linking mediated by LOX has consistently been found in several human fibrotic disorders, such as hypertrophic scar and liver fibrosis [152–156]. This observation suggests that collagen containing hydroxyallysine cross-links is less susceptible to proteolytic degradation and therefore the collagen deposition is no longer reversible.

Recent studies have demonstrated that fibrosis can be halted or even reversed depending upon the extent of its progression [157,158]. Currently, the regression of fibrosis has been studied mostly in the liver [159,160]. It is associated with termination of the chronic injury, loss of TGF-β1 signaling, and decrease of pro-inflammatory cytokines
Recruitment of myelo-monocytic cells to the injured organ is critical for the resolution of fibrosis [162]. Inflammatory cells not only participate in clearance of collagen-producing cells, but also secrete matrix metalloproteinases (MMPs), which play a crucial role in ECM remodeling and regression of fibrosis. However, advanced cirrhosis in liver is resistant to collagenolysis due to formation of irreversible non-reducible crosslinked collagen and an ECM enriched with elastin fibers preventing its degradation [163]. This pathophysiologic state is considered the “‘point of no return’” [163]. Severe damage of the liver tissue resulting from the loss of the integrity of the basement membrane due to the progressive deposition of collagen matrices in the space of Disse, increased intrahepatic resistance to blood flow and portal hypertension finally lead to hepatocellular carcinoma and that may also prevent resolution of fibrosis [164].

Since the contractile stresses of the surrounding microenvironment in fibrotic disease progression are a key mediator in the differentiation of myofibroblasts, it is suggested that alteration in the ECM biomechanical properties, stiffness in particular may be an important therapeutic target that is able to modulate myofibroblast formation and fibrosis [111,165]. Recent studies suggests that when valvular, liver, or lung fibroblasts are cultured on low modulus substrates (E \leq 10 \text{kPA}), they maintain a normal phenotype; however, they are activated to myofibroblasts when they are cultured on higher modulus substrates [111,165–168]. Marinkovic et al. tested whether matrix stiffness can control the function of fibroblasts derived from idiopathic pulmonary fibrosis compared with fibroblasts derived from normal lung tissue in collagen hydrogel substrates with the stiffness spanning from normal and fibrotic lung tissues [111]. They demonstrated that
the contractile and proliferative function in primary fibroblasts derived from fibrotic lungs were significantly inhibited when they were cultured in soft matrices (~1 kPa of elastic modulus), which is the physiological level of lung ECM stiffness, suggesting that the myofibroblast phenotype is not a permanent state but can be reversed by alterations in the matrix properties. Wang et al. utilized a photodegradable poly (ethylene glycol) (PD-PEG) hydrogel to study the fate of valvular myofibroblasts in response to reduced substrate modulus [165]. Their results indicated that valvular myofibroblasts grown on soft substrates (7 kPa, mimicking healthy cardiac valve fibrosa) show a decrease in α-smooth muscle actin (α-SMA) stress fibers and proliferation and an increase in apoptosis, while the levels of gene expression including α-SMA and CTGF were significantly up-regulated when valvular myofibroblasts were cultured on stiff substrates (32 kPa, mimicking pre-calcified diseased tissue). This study suggests that the mechanical stiffness of the substrates can regulate the fate of activated myofibroblasts, resulting in a predominantly quiescent fibroblast population. Thus, understanding the plasticity of the fibrotic phenotype is critical to development of novel therapeutic approaches to fibrosis.

1.4. Current Antifibrotic Therapy and Limitation

The increasing evidence that fibrosis is a dynamic and reversible process, the clarification of the underlying mediators of fibrosis progression, and advances in non-invasively assessing fibrosis have generated enthusiasm towards developing effective antifibrotic drugs. To date, however, no drug has been approved as a clinically safe and effective antifibrotic [169]. Challenges remain including the lack of the etiology and pathogenesis due to the diversity and heterogeneity of fibrotic diseases and suitable
surrogate parameters capable of measuring the effectiveness of novel therapeutic agents. Here, recently ongoing studies regarding interference with TGF-β expression and activation using small molecules or neutralizing antibodies are summarized.

1.4.1. Interfering with TGF-β Expression and Activation

As described earlier, TGF-β is the most important causative agent involved in the fibroblast activation process as well as the mesenchymal transformation of epithelial and endothelial cells [170]. Thus, preventing TGF-β signaling is clearly a potential therapeutic approach that may be achieved at several levels. First, the complex signaling pathways mediated by TGF-β receptor binding offer multiple points of potential therapeutic intervention. TGF-β is also constitutively synthesized and stored in an inactive form as a complex with specific binding proteins. Thus, the expression of TGF-β and the process of its release in an active form represent promising therapeutic targets.

Pirfenidone (5-Methyl-1-phenyl-2-(1H)-pyridone), marketed under the names Esbriet and Pirespa is a small orally bio-available molecule that is the first targeted antifibrotic drug to be approved for the treatment of IPF in Europe and Japan [84,169]. Pirfenidone exhibits antifibrotic and anti-inflammatory properties in a variety of in vitro and animal models [171–173]. In vitro studies have demonstrated that pirfenidone inhibits TGF-β-induced collagen synthesis [171,172], decreases ECM deposition and blocks the mitogenic effects of PDGF in lung fibroblasts derived from patients with IPF [173]. Pirfenidone has also shown broad antifibrotic activity in several animal models of fibrosis in lung and other organs. In pulmonary fibrosis study, pirfenidone reduced fibrosis in response to bleomycin, lung transplant and repeated allergen exposure through
the suppression of TGF-β gene expression and significantly reduced the synthesis of collagen type I and III. Pirfenidone has been extensively evaluated through open-labeled compassionate use studies followed by four randomized, double-blind, placebo-controlled clinical trials, phase III studies. The almost identical multinational 004 and 006 trial studies were conducted, and the third trial was performed in Japan [174,175]. In the 004 trial, 435 patients with IPF were assigned in a 2:1:2 dosing ratio to 2,403 mg/day pirfenidone, 1,197 mg/day prifenidone, and placebo. In the 006 study, 344 patients were assigned to either 2,403 mg/day of pirfenidone or to placebo. In study 004, pirfenidone-treated patients exhibited increased forced vital capacity (FVC) (P = 0.001) compared with the placebo group (difference 4.4%, 95% CI, 0.7 to 9.1), however in study 006, the change in FVC at 72 weeks was not significant between the treatment and placebo groups (P = 0.501). In the double-blind, placebo-controlled randomized clinical trial conducted by Taniguchi et al., pirfenidone was administered in a 2:1:2 ratio (1,800 mg/day, 1,200 mg/day or placebo) to a total of 275 patients over a 52 week period [174]. The primary endpoint, a change in lung vital capacity, was significantly preserved in the higher dose versus placebo group (–0.09 vs. –0.16 L respectively, P = 0.0416). Limitations to this study include the enrollment of a relatively homogeneous Japanese population, as well as the fact that the primary end-point was changed before unblinding. In an exploratory analysis of this study later published by Azuma et al., it was observed that a subpopulation of these patients had a greater benefit from pirfenidone [176]. A well-known side effect of pirfenidone, photosensitivity, was also frequently observed in this study (51% of patients in the high-dose group and 53% in the low-dose group). Despite
an approval by both the EU and Japan, due to the lack of therapeutic efficacy of pirfenidone in FVC and survival benefit, the use of pirfenidone for treating pulmonary fibrotic diseases has not been approved by the FDA. A new phase III trial of pirfenidone aiming to detect a clinically meaningful effect on FVC is therefore underway in the United States (the ASCEND trial).

Many neutralizing antibodies to TGF-βs have been successfully shown to prevent fibrosis in a number of organs in animal models through their direct binding to TGF-β ligands [177]. Due to TGF-β’s pleiotropic, multiple roles in immunomodulation, tumor suppression, and wound healing, the broad targeting of TGF-β ligand to treat chronic disease in humans may have detrimental side effects [34,178,179]. For example, inhibition of TGF-β receptor type I prevented the overexpression of collagen type I and enhanced ECM contraction by fibrotic dermal fibroblasts isolated from scars of scleroderma patients. However, it also significantly affected basal collagen type I production and ECM contraction by normal fibroblasts. In addition, one patient developed a premalignant skin lesion while receiving a TβRII antibody, but upon discontinuation of the drug, this effect resolved with time. Moreover, TGF-β is known to stimulate angiogenesis by upregulation of VEGF, which can be blocked by a treatment with TGF-β neutralizing antibodies. Furthermore, the pre-clinical success of TGF-β-targeting antibodies has not translated into clinical efficacy. First, the human monoclonal antibody metelimumab (also known as CAT-192) was clinically investigated and compared with placebo in 45 patients with early systemic sclerosis. In this clinical trial, the antibody was given by intravenous infusion at baseline and at weeks 6, 12 and 18, and
patients were evaluated at 24 weeks. The trial showed no improvements in skin scores and other disease manifestations in patients treated with CAT-192. Limitations of the study include the restricted isotype specificity of the antibody and its low binding affinity, the short treatment duration and small number of patients. A monoclonal neutralizing antibody to TGF-β2 (lerdelimumab, or CAT-152) has also been tested for the prevention of scarring following glaucoma surgery. In addition, a monoclonal antibody (GC1008) targeting all three TGF-β isoforms is currently in a phase I trial for treating idiopathic pulmonary fibrosis. However, results to date in humans have not been promising.

1.4.2. Blocking TGF-β Signaling Pathways including TGF-β receptors and SMAD molecules

There have been several studies of specific inhibitors of TGF-β type 1 receptor and the most thoroughly studied is SM305 that proved to have excellent selectivity and potency against ALK5 and ALK4. In normal dermal fibroblasts, SM305 abrogated TGF-β-induced ECM gene expression, fibrogenic cytokine production, Smad3- and Smad2-dependent transcriptional responses, and fibroblast transdifferentiation into myofibroblasts. These inhibitory effects of SM305 were associated with potent selective suppression of TGF-β-induced phosphorylation and nuclear translocation of R-Smads. However, in unaffected SSc fibroblasts, SM305 only caused variable and modest reduction in levels of type I collagen, and did not reverse constitutive Smad nuclear accumulation or the proportion of α-SMA-positive myofibroblasts. The contradictory results with SM305 in SSc fibroblasts therefore indicate that the suitability of the kinase
inhibitor to target TGF-β must be carefully considered due to complexity and heterogeneity of fibrotic reactions and thoroughly evaluated in animal models and ultimately, possibly in humans.

Preventing TGF-β action is clearly a promising therapeutic approach for many fibrotic diseases, however, evidence from the clinical applications revealed undesired side effects due to the multi-functional activities of TGF-β and consequent systemic side effects [126,179–181]. Therefore, the development of novel antifibrotic therapies is desperately needed.

1.5. Mechanotherapy

Cells and tissues are capable of responding and adapting to their mechanical environment. Mechanical forces direct cellular activities influencing the tissue-level processes of growth, remodeling, and repair, with the ultimate outcomes being altered tissue mass, structure, and function. In 1890, mechanotherapy was first defined as “the employment of mechanical means for the cure of disease” [182]. Typical examples of classical physical therapies are massage and orthopedic rehabilitation that aim to promote symptom relief or functional recovery towards pre-surgical levels with or without the help of specific operational equipment or devices [183]. More recently, the definition of mechanotherapy has expanded to include “any intervention that introduces mechanical forces with the goal of altering molecular pathways and inducing a cellular response that enhances tissue repair and remodeling” [184]. Thus, mechanotherapy describes a group of therapeutic interventions intended to reduce and reverse injury to damaged tissues or
promote the homeostasis of healthy tissues by mechanical means at the molecular, cellular, or tissue level [183–185].

1.5.1. Mechanotransduction

Mechanobiology is an interdisciplinary field that investigates cells’ biological responses to mechanical stresses and the mechanotransduction pathways by which these loads are transduced into a series of cellular and molecular events [186]. Mechanotransduction is generally broken down into four phases: (1) the mechanocoupling phase, where the external mechanical signal is converted into a mechanical signal in the vicinity of the cell; (2) biochemical coupling, where the local mechanical signal is transduced into a biochemical signal, resulting ultimately in genetic or protein changes; (3) signal transmission, where the biochemical signal is then passed from the sensor cells to the effector cells; and (4) the effector cell response [183,184].

When the cells are exposed to a variety of micromechanical stimuli such as tension, compression, shear, hydrostatic pressure, vibration, and fluid shear, transmembrane integrins transfer mechanical forces from the extracellular matrix (ECM) to the cytoskeleton through focal adhesion complexes. This activates signal transduction cascades, which in turn alter cytoskeletal functions and induce ECM remodeling. The actin cytoskeleton can thus act as a ‘global mechanical signal integrator’ [183].

The conversion of mechanical stimuli to the mechanochemical signal can be explained through the tensegrity networks proposed by Ingber et al [187]. The tensegrity architecture of the cytoskeleton is a self-assembling system driven by structural hierarchies and the tensile stresses of the cell that yields a dynamic balance between
counter-acting forces of compression and tension, then finally leading to a self-equilibrated mechanical stability [188,189]. Within a tensegrity network, mechanical forces received by the cytoskeleton of a cell from the ECM not only change the cell that receives the signals but are also transferred to neighboring cells through cadherin-mediated cell–cell adhesion complexes. Thus, cells connected to each other and to the ECM form a dynamic network that can be manipulated by externally applied mechanical stimuli. Based upon the recognition that a wide range of intracellular signaling molecules and enzymes bind to the actin cytoskeleton, Ingber and co-workers have proposed a theory of ‘solid-state biochemistry’ for mechanical-chemical conversion within cellular tensegrity structures in which cytoskeletal re-arrangements in response to mechanical forces create changes in enzyme conformation/activity and enzyme/substrate proximity [190].

Several researchers also highlight the transcriptional regulation by cytoskeletal forces inside the nucleus [191–193]. Cytoskeletal filaments are connected to the nuclear membrane through the linker of nucleoskeleton and cytoskeleton complex (LINC complex). Within the complex, a family of nesprin and SUN-domain proteins play an important role in connecting the actin cytoskeleton with the lamina nucleoskeleton [191,194,195]. Nesprins 1 and 2 bind actin filaments, nesprin 3 binds plectin associated with intermediate filaments, and nesprin 4 interacts with microtubule networks through the molecular motor kinesin 1. The C-terminus of nesprin proteins contains a KASH domain that binds SUN1 and SUN2, and SUN domain is located in the nuclear envelope [196]. The N-terminus domain of SUN proteins binds to lamins and other structural
proteins of the nucleoskeleton [194]. The lamin proteins can bind to DNA and chromatin through complexes of lamin binding proteins and other structural proteins, including transcriptional repressors and regulators. Thus, externally applied mechanical stimuli transmit from the ECM through the cytoskeleton via integrin-mediated interactions and then finally into DNA and chromatin bound to the nucleoskeleton through the LINC complex.

Of particular interest currently in integrin-mediated mechanisms is the transient receptor potential (TRP) superfamily that are mechanosensitive ion channels that are activated by multiple endogenous and external stimuli to mediate a wide variety of mechanotransduction processes in diverse organs and species [197–200]. The TRP superfamily is composed of 28 different genes that are divided into seven different subfamilies (TRPA, TRPC, TRPM, TRPML, TRPN, TRPP, and TRPV). They are known to be activated by the following: 1) direct ligand binding, 2) depletion of intracellular Ca\textsuperscript{2+} store and Ca\textsuperscript{2+}/calmodulin-dependent activation, and 3) indirect activation by osmotic stress, temperature variation, pheromones, taste, and mechanical as well as other stimuli [199,201]. In particular, there are two models (direct or indirect) for activation of TRP ion channels by mechanical stimuli [202,203]: a) direct activation by force conveyed through externally stretched lipid, b) direct activation by force conveyed through linker proteins that are bound to both TRP channel proteins and adhesive proteins such as intracellular cytoskeletal elements and/or extracellular matrix molecules, c) indirect activation by force conveyed to accessory proteins that are anchored to channel proteins. The accessory proteins are bound to a mechanically sensitive proteins
that carry the signal into a ligand-activated channel, and d) indirect activation by a secondary signal such as a diffusible second-messenger molecule or activation of a kinase generated by a force-sensing protein complex. Resulting from these changes, the TRP ion channels open or close, allowing the influx or efflux of potassium and calcium ions, leading to alteration in molecular and cellular functions.

Furthermore, cells receive these exogenous forces through interaction with the ECM, and the stiffness of the ECM substrates is an important mechanical determinant of cell behavior [204]. Several studies also suggest that the alteration in substrate stiffness affects cellular function and changes the structure and composition of the ECM, eventually leading to the changes in tissue development, homeostasis, and wound healing [205–214]. Flanagan et al. developed the protein-laminated polyacrylamide gels with varying amounts of bisacrylamide to generate substrates with variable elastic moduli. Mouse primary neuronal cells cultured on softer substrates (E ~ 0.1–1 kPa) showed threefold increased formation of neurite branches compared with those grown on stiffer gels [215]. Engler et al. used micropatterned collagen-coated poly-acrylamide (PA) gel substrates to investigate the effect of matrix stiffness in striated muscle differentiation [216]. 4 weeks after plating myoblasts on collagen-coated PA gels of varied stiffness, myoblasts cultured on the matrices that mimic striated muscle like-elasticity (E ~ 8–17 kPa, intermediate stiffness) showed significant myosin striation and myofibril maturation compared with the cells grown on soft or rigid substrates. Cells on rigid substrates produced well-formed stress fibers and numerous vinculin-enriched focal adhesions, but did not achieve actin reorganization into myosin striations. On the other hand, cells on
soft substrate did not form strong adhesion and contractile forces, resulting in poorly-organized inter-cytoskeletal structures and lack of striations. In addition, osteoblast differentiation from human mesenchymal stem cells has been shown to occur on stiffer substrates (E ~ 25–40 kPa) that mimic the cross-linked collagen of osteoid [217–219]. These results show that mechanical properties of the substrate specifically direct specific lineage differentiation of mesenchymal stem cells, which are critical for tissue development and regeneration. Thus, understanding of how biomechanical forces can be transmitted and influence cell behaviors will also help elucidate the pathogenesis of many diseases [177,220,221], and hence provide new mechano-therapeutic approaches to treat these pathological conditions more effectively.

1.5.2. Effect of Mechanical Stimulation on ECM Remodeling-related Molecules

Mechanical forces are essential regulators of tissue homeostasis and development and indispensable for normal function, particularly of connective tissues comprised mainly of fibroblasts, as they are subjected to various forms of mechanical loadings such as tension, compression, and shear stress in the organism. It has been demonstrated that mechanical forces specifically regulate the synthesis and degradation of various ECM components such as collagen, GAGs and proteoglycans. Since the first study illustrating the effect of mechanical loading on ECM production was reported by Leung et al. [222], numerous studies have shown that mechanical loading is able to affect fibrous procollagen expression depending on the cell source and loading conditions [221,223]. For example, compared to the unstretched cells, a single application of a 10% uniaxial stretch resulted in a threefold increase in collagen type I, III and fibronectin mRNA levels.
in cardiac fibroblasts [224]. However, when cardiac fibroblasts were stretched for 20%, a significant inhibition of ECM-related genes was observed [225].

In response to cyclic strain, anterior cruciate ligament (ACL) and medial collateral ligament (MCL) fibroblasts have shown a differential mRNA expression in collagens type I and III after stretching [226]. ACL fibroblasts exhibited higher levels of type I collagen mRNA with no apparent changes in type III collagen mRNA, whereas MCL fibroblasts responded to cyclic strain by a significant increase in type III collagen, but not in type I collagen mRNA. Interestingly, in contrast to many other studies, Jiang et al. reported that human skin fibroblasts grown on mechanical stretch device with 20% cyclic stretching and 1 Hz frequency for 6 days showed a significant reduction in collagen type I expression, but also observed a significant increase of collagen type I in response to non-stretch and static stretch conditions [227].

Connective tissue cells have also been found to be responsive to the characteristics of applied loading, including strain magnitude and frequency. In human tendon fibroblasts, when cells were subjected to uniaxial stretching with constant frequency and duration (0.5 Hz, 4 hr), but various magnitudes of stretching (~ 4–8%), cell proliferation, collagen type I gene expression, and protein production all increased in a stretch magnitude-dependent manner [228]. Periodontal ligament fibroblasts (PDLFs) exhibit differential responses to varying levels of mechanical strain as well. A 5% cyclic stretch for 24 hours increased synthesis of both collagen type I (twofold) and fibronectin (threefold) [229]. However, exposure to a 10% strain exhibited a similar response for fibronectin (fivefold increase), whereas the amount of type I collagen synthesized by the
stretched cells did not differ from unstretched control levels, showing that these cells are capable of modifying their responses to varying magnitudes of tensional stress. In addition, adult cardiac fibroblasts also show differential responses to different stretching magnitudes. A cyclic tensile strain at 3% increased mRNA expression levels of collagen type III and fibronectin by 1.5-fold; however, 6% stretching decreased collagen type III mRNA levels while fibronectin levels remained unchanged [225,230]. Furthermore, the effect of cyclic stretching variables such as strain, pulse shape, and pulse frequency has been reported for human dermal fibroblasts [231]. Joshi and Webb examined dose-dependent responses of cell/scaffold stiffness to systematic variation in cyclic parameters including strain amplitude, rate, frequency, and duration, and demonstrated that varying cyclic strain regimes differentially regulated construct mechanical properties [231].

Concomitant with increased expression of collagen type I in response to mechanical loads, numerous studies have shown that mechanical stress also stimulates increased expression of TGF-β1 in a number of cell and tissue types [228,232–237], which in turn serves as an underlying mechanism for strain-mediated effects on ECM expression, particularly collagen synthesis and accumulation. Several studies indicated that collagen α1 type 1 gene expression induced by mechanical stress is mediated by the autocrine or paracrine release of transforming growth factor (TGF)-β [232,238–241]. In addition, mechanical loadings can lead to the activation of pre-existing TGF-β in the ECM of myofibroblasts that can stimulate the target genes such as procollagen α1 type 1 [242]. When the fibroblasts were subjected to mechanical stress, not only the secretion of active TGF-β is stimulated, but also the mRNA expression of TGF-β is enhanced via
activation of EGR-1 (Early growth response protein 1) which has three zinc finger motifs to bind and regulate transcription in many growth factors and cytokines [243]. Specifically, with regard to fibroblasts, tendon fibroblasts cultured under cyclic uniaxial stretching (4% and 8% constant stretching at 0.5 Hz for 4 h) showed increased expression of TGF-β compared to that of non-stretched fibroblasts [228]. Also, the increased expression of TGF-β1 in response to cyclic strain at 1 Hz (per cycle 0.5 sec elongation and 0.5 sec relaxation) for 4 h has been reported for cardiac fibroblasts [244]. Cyclic stretching increased levels of TGF-β1 by 21 ± 4% upon 4 h of cyclic stretching. Furthermore, the mRNA synthesis and protein production of CTGF are also induced by mechanical stress [245]. CTGF expression was upregulated by more than twofold in 4 h and remained elevated at this level after 8 h, when mesangial cells are subjected to cyclic mechanical strain [246]. Schild and Trueb also noted a substantial increase (fourfold) in the levels of CTGF mRNA in mechanically stressed fibroblasts [245].

Additionally, recent studies have shown that mechanical loading of cells also modulates ECM turnover by regulating the expression of MMPs and their activity. Human dermal fibroblasts cultured under cyclic stretching (20%) at low frequencies (0.1 Hz) for 24 h produced a significant increase in MMP1 and type I collagen mRNA levels compared to nonstretched controls. TIMP1 synthesis was significantly induced by mechanical tension compared with unloaded control at 72 h to 96 h of culture. The results demonstrated that the level of TIMP1 increased 63.9% at 24 h; and 64.42%, 57.1%, and 69.9% at 48 h, 72 h and 96 h of culture, respectively [247]. In addition, cDNA microarray study performed by Kessler et al. demonstrated that human dermal fibroblasts cultured
within three-dimensional collagen networks under stressed condition exhibited significant increase in the MMP1 and tissue inhibitors of matrix metalloproteinases (such as TIMP1 and TIMP3) compared with relaxed conditions [38].

1.5.3. Current Mechanotherapy in Wound Healing

Mechanotherapies focusing on improving wound healing include negative pressure wound therapy (NPWT), shockwave therapy and surgical tension reduction. First, NPWT is known clinically as vacuum-assisted closure (VAC) and involves the application of a vacuum to a wound surface by sealing the wound with a porous polyurethane sponge and occlusive dressing connected to the vacuum [248]. It is effective for treating acute wounds as well as chronic, open, large, and contaminated wounds [249], because it can remove extracellular fluid, stabilize the wound environment, generate contracture of the wound or macrodeformation, and induce microdeformation at the foam–wound interface in the wound area [250]. In addition, it has been shown to promote angiogenesis through increased VEGF production [251,252]. Furthermore, there is in vitro evidence showing that NPWT of human dermal fibroblasts in a provisional wound matrix elevates the concentrations of TGF-β, platelet-derived growth factor-α (PDGF-α), and PDGF-β [253]. Similarly, mouse dermal fibroblasts treated by a suction/foam/perfusion bioreactor in vitro exhibit upregulation of basic fibroblast growth factor (bFGF), TGF-β1, Type I collagen α1, and smooth muscle actin α2 mRNA expression [254].

Extracorporeal shock wave therapy (ESWT) is currently being investigated to enhance wound healing. This approach involves biphasic high-energy acoustic waves that
can be generated by electrohydraulic, electromagnetic, or piezoelectric technologies. Clinical outcomes suggest that ESWT is effective in both diabetic [255] and surgical wounds [256] by reduced wound size [257], enhanced re-epithelialization, neovascularization, and blood perfusion [258–260], and decreased pain and necrosis [261,262]. At the molecular level, shockwave therapy upregulates TGF-β1 expression in fibroblasts [263] and suppresses the production of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α [264]. It also triggers anti-inflammatory activity by, for example, increasing neuronal NO synthase (nNOS) activity and NO production in the C6 rat glioma cells while concomitantly downregulating NF-κB and TNF-α gene expression [265]. Shockwave therapy has been recently investigated as an adjuvant therapy in the treatment of acute and chronic wounds [266].

In addition, mechanotherapies are highly useful for preventing and treating pathological scars and reducing their recurrence. The association of pathological scars with skin tension has been well-documented: most of these scars occur in areas of the body that are subjected to frequent mobility and/or high stretching tension [267]. Significantly, mechanical stress applied to an injured area produces hypertrophic scars in mice [268]. These destructive local mechanical forces on the wound can be alleviated by employing refined surgical tension-reducing techniques, such as a small-wave incision design [269], local flaps to cover the wound, silicon sheeting [270], and subcutaneous/fascial sutures [271]. Recently, a stress-shielding technique based on a dynamic polymeric device reduced the histological scar area of incisions in swine by 9-fold compared with incisions in a stressed state; a subsequent Phase I clinical study
showed that the stress-shielding device decreased hypertrophic scar formation in humans with high-tension abdominoplasty incisional wounds that are prone to excess scarring [272].

It is widely noted that mechanical forces can activate intercellular biochemical signals through the mechano-responsive transmembrane and ECM molecules and then lead to molecular and cellular responses via altered activation of downstream signaling pathways. Despite successful clinical outcomes in orthopedic and wound healing practices, more in-depth discussion on the intracellular molecules and molecular signaling pathways responsive to mechanical stress are needed. In addition, huge challenging questions remain in the developments of mechanotherapy, particularly specificity, selectivity, and timeliness with regard to the application of mechanical forces. More specifically, dosing parameters such as type, amplitude, duration, and frequency need to be carefully investigated to make a novel therapy more specific and effective [183].

1.6. Vocal Folds

1.6.1. Vocal Fold ECM and Biomechanics

Voiced sound is produced by vibration of the vocal folds, which converts aerodynamic energy from exhalation to acoustic energy. The unique biomechanical properties of the vocal folds result from its distinctive, hierarchal ECM composition and organization that allow them to undergo high frequency oscillations. The vocal folds are stretched across the larynx located between the trachea and the pharynx. Each fold has a length of 10–20 mm along the anterior-posterior direction and a thickness of 3–10 mm.
The human vocal folds can be divided broadly, anatomically into three tissue layers: stratified squamous epithelium, lamina propria, and vocalis muscle anchored to the thyroid and arytenoid cartilages.

Hirano et al. first subdivided the vocal fold lamina propria into superficial, intermediate, and deep layers based upon histological composition [273–277]. The epithelium and the superficial layer of lamina propria comprise vocal mucosa. The gelatinous superficial layer (Reinke's space) of the lamina propria has a small number of cells and is composed of loosely clustered reticular collagen fibers and small, amorphous forms of elastin fibers arranged in a longitudinal directions. This layer functions as a pliant cushion during vocal fold phonatory cycles, providing resistance to impact stresses during phonation [278,279]. Anchoring looped collagen fibers in the superficial lamina propria form an intertwined network, which is highly branched, delicate three-dimensional matrix filled with large amount of glycoproteins, GAGs, and elastic fibers. The vocal ligament contains the intermediate and deep layers of the lamina propria. The intermediate layer is composed of mostly mature elastin fibers arranged in parallel to the edge line of the vocal fold. This layer provides elastic mechanical integrity to the vocal fold. Finally, the deep layer of the lamina propria is composed entirely of mature collagen fibers and coiled elastin. With regard to the ECM distribution, collagen types I and III generally exist in all of the layers in the vocal folds, whereas collagen type III is dominant in the lamina propria, particularly in the intermediate and deep layers of vocal folds [280]. Munoz-Pinto et al. demonstrated that the average thickness and angular deviations of collagen fibers increase with depth toward the thyroarytenoid muscle [281].
Elastin that contributes to tissue pliability and elasticity is located in the intermediate layer of vocal fold lamina propria at highest density [282]. Hyaluronic acid (HA) is highly concentrated in the intermediate layer of vocal folds and provides the gel-like and cushioning characteristic to the structure [283]. Decorin and fibromodulin, small proteoglycans found primarily in the superficial layer of the lamina propria are known to bind collagens and regulate the collagen fibril formation in vocal fold tissue [284]. The unique arrangement of vocal fold ECM, therefore, allows the vocal folds to resist longitudinal forces during phonation, while permitting mucosal vibration in the lateral plane.

Voice production is initiated and regulated by the coordination of many different factors, including respiration (air pressure and flow), phonation (vocal fold biomechanical property), and resonance (the cavities above and below the glottis) [285]. During exhalation, the air coming out from the lungs encounters the vocal folds postured at the midline. As the pressure and flow of the air rise above the phonation threshold pressure (PTP), air begins to pass through the glottis. At this point, the elastic properties of the vocal ligaments and the Bernoulli’s forces generated by the air pressure difference between the cavities above and below the vocal fold tissues bring vocal folds to the midline. The vocal folds, thus, open and close repeatedly, resulted in the production of vibratory patterns at frequencies of around 200–220 Hz for women and 100–120 Hz for men. This rapid vibration of the vocal folds generated by repetitive vibratory cycles produces “voiced sound” after amplification and modification by the vocal tract resonators [286].
The cover-body theory of vocal fold vibration originated by Hirano and Kakita proposed that the relatively compliant vocal mucosa (cover) oscillates over the relatively stiff vocalis muscle (body) along with the vocal ligament serving as a transition zone [287]. The vibration of the vocal fold mucosa is characterized as a mucosal wave that propagates in the horizontal, longitudinal, and vertical planes at physiological frequencies ranging from 100 to 300 Hz and amplitudes of 1–2 mm during normal phonation [288]. On the other hand, the vocal ligament oscillates at lower frequencies of 1–10 Hz and experiences maximum tensile strains estimated to range from 15–60% [289,290].

Vocal quality depends on the vocal fold extracellular matrix (ECM) composition and organization. With regard to age, the distinctive multi-layer structure of vocal folds was not clearly observed in newborn vocal folds [291,292]. As the vocal folds undergo substantial growth and maturation during adolescence, the unique matrix composition and multi-layered formation of lamina propria were identified between ages 13 through 17 [292,293]. In addition, biomechanical and compositional changes in vocal folds have been reported with respect to gender. The levels of hyaluronic acid in lamina propria are about three times greater in men than those in women, which is observed by increased lamina propria thickness for men [294]. Furthermore, increased elasticity and viscosity are observed in men and geriatric patients relative to women and younger patients [295–297].

Regarding vocal fold injury, excessive mechanical stresses imposed by prolonged phonation, a complication of surgical procedures, or chemical damages can lead to vocal fold scarring. Histological examination has reported compositional alterations after vocal
fold injury, including increased type I and III collagens, increased fibronectin deposition and decreased elastin density and organization [298]. The consequence includes incomplete generation of vibration caused by imperfect vocal fold closure during phonatory cycles. Thus, based on previously published studies and observations, the complete biomechanical function of the vocal folds is supported and maintained by the dynamic interactions of its unique matrix components.

1.6.2. Scarless Wound Healing of Vocal Folds

Vocal fold damage resulting from a multitude of factors, such as traumatic injury, intubation, phonotrauma, gastroesophageal reflux disease, chemical irritation, and the surgical treatment of benign, chronic infections ultimately leads to complete loss of vocal function. Although the regenerative capability of the vocal folds is clearly limited depending on the magnitude of tissue injury, several researchers highlight the anti-scarring property of vocal fold lamina propria. Based on the histological observation reported by several researchers, the presence of myofibroblasts and macrophages in healthy vocal tissue in the superficial lamina propria layer suggested that the mechanical stresses imposed during normal phonation result in repetitive microtrauma that is repaired without significant compositional, structural, and functional losses [284,293,299,300]. Although the mechanisms underlying scarless repair of vocal fold injury in the LPs have not been fully elucidated, several studies suggest that the ECM components of the vocal fold SLLP provides resistance to scar formation. For example, hyaluronic acid (HA) is known to play an important role not only in proper vocal fold vibration by contributing to the viscoelastic properties of the vocal fold cover as a space filling material, but also in
the acute phase of wound healing. For example, HA has been shown to inhibit fibroblast collagen synthesis and deposition [301,302]. Human dermal fibroblasts cultured in the media with the addition of HA (0.5 and 1 µM) showed decreased production of ECM, particularly collagen synthesis, compared with the collagen levels produced by fibroblasts cultured in the absence of HA supplementation. They also indicated that high concentrations of HA in the ECM during wound healing reduced the deposition of ECM proteins, thus resulting in the wound healing process with less scar formation [302]. Furthermore, several studies indicated that two small proteoglycans: decorin and fibromodulin, predominantly located in the superficial layer of the lamina propria (SLLP) are associated with anti-scarring activity. Both molecules are known to bind collagen, alter the kinetics of fibril formation [303], and inhibit fibrillogenesis [303–305]. Decreased levels of both decorin and fibromodulin have been observed in the fibrotic scar formation in the skin [280,306]. Reduced levels of decorin and fibromodulin may contribute to the altered collagen structure associated with vocal fold scarring. Moreover, both proteoglycans have been shown to bind and sequester TGF-β1, a potent fibrotic cytokine that stimulates collagen synthesis during scarring and fibrotic disease [278,304,307–311]. Decreased TGF-β-mediated matrix accumulation and glomerular injury were also reported after the addition of decorin to a rat model of glomerulonephritis [312].

Many studies demonstrated that the anti-scarring property of vocal fold lamina propria resulted from its unique matrix composition. Several lines of evidence also showed that matrix composition can be modulated and regulated by mechanical forces
through mechanotransduction pathway. Therefore, since the vocal folds extensively experience unique mechanical stresses, we have questioned whether vibratory stimulation can provide the resistance to vocal fold scarring via activation of altered downstream signaling molecules and pathways.
2.1. Specific Aims

Fibrosis is a complex disease predominantly characterized by excessive and abnormal fibrous ECM deposition, leading to the failure of various organs: lung, liver, kidney and skin. During the normal wound healing process, activated myofibroblasts migrate into the wound area, where they rapidly synthesize and remodel newly created ECM to maintain tissue integrity during repair. Fibrotic disorders result from dysregulation of this process, commonly involving the abnormal persistence of several pro-fibrotic cytokines that leads to sustained myofibroblast activation and ongoing ECM synthesis. Specifically, TGF-β is considered a master switch that activates critical downstream molecules in the progression of fibrotic disease [24,123–126,133,159]. Although various strategies designed to interfere with TGF-β expression, receptor binding, and signal transduction have been studied, a clinically safe and effective therapy has not yet been developed.

Recently, many researchers highlight mechanical forces as therapeutic tools, especially focusing on how mechanical forces can be applied to promote wound healing or reverse pathological processes. Mechanical stimulation plays a central role in regulating ECM composition and organization and cytokine production during development and wound healing. Vocal fold tissues differ from other connective tissues in terms of their unique biomechanical and biochemical properties. The vocal mucosa (epithelium and superficial layer of lamina propria (SLLP) is continuously exposed to
high frequency vibration, while the vocal ligament (intermediate and deep layers of lamina propria) experiences lower frequency cyclic tension. Collagen density increases with depth in the lamina propria, suggesting that differences in the mechanical microenvironment are able to stimulate differential expression of ECM-related genes. The presence of macrophages/myofibroblasts in the SLLP of healthy patients suggests that vibratory stress combined with impact stress when the two folds collide at the laryngeal midline results in repetitive microtrauma. However, the limited amount of mature collagen further suggests that the SLLP possesses the capability to continuously repair this damage without permanent scarring. Therefore, the objective of this research is to understand the mechanisms regulating fibroblast matrix metabolism in the SLLP and investigate the potential of vibratory stimulation for treatment of fibrotic diseases. The specific aims are:

Aim 1: To characterize transcriptional and translational response of human dermal fibroblasts activated by vibratory stimulation. Vibratory stimulation has been reported to increase the expression of matrix-related genes consistent with the native vocal fold tissue physiology and potentially provide resistance to scarring, including down-regulation of type I collagen. This study is the first to characterize the comprehensive transcriptional response of human dermal fibroblasts to high frequency vibration and to identify down-regulation of the TGF-β signaling pathway triggered by vibratory stimulation. Normal human dermal fibroblasts (NHDF) seeded in porous polyurethane substrates were subjected to either: 1) further static culture (Static), 2) one-time 20% axial strain followed by static culture (1X Strain), or 3) one-time 20% axial strain
followed by vibration (1X Strain + Vibration, 100 Hz frequency, 3.8 Vrms, 1.28 ± 0.07 mm amplitude). Microarray analysis was conducted to examine the transcriptional response caused by vibratory mechanical stimulation. qRT-PCR and ELISA assays were then performed to validate the microarray results for cytokine and collagen expression at the 6 h time point and investigate if these changes were maintained up to 7 days. The effects of vibratory stimulation on collagen deposition and mechanical stiffening of polyurethane substrates were evaluated using two in vitro fibrotic models.

Aim 2: To characterize the effect of different vibratory regimes on fibroblast matrix synthesis. Vibratory stimulation is characterized by three principle characteristics (frequency, amplitude, and duration). Biomechanically, vocal fold mucosa experiences vibration at high frequency (100–200 Hz) and oscillates approximately 1 mm laterally during each vibration cycle. Many mechanical stimuli are known to elicit dose-dependent responses, including varying levels of increased or decreased expression of genes and proteins, collagen production, and mechanical stiffness. The goal of this study was to investigate the effect of variable vibratory frequency, amplitude, and duration on the expression of human dermal fibroblast ECM-related genes by quantitative real-time RT-PCR. Thus, this study will allow complete understanding of the relationship between variable parameters and responses, providing a first step towards identification of optimal treatments for vibratory therapy.

Aim 3. To evaluate the potential therapeutic efficacy of vibration for reversing the fibrotic phenotype in scleroderma-derived fibroblasts. The fibroblasts from patients with systemic sclerosis (SSc) are known to exhibit an abnormal phenotype with expression of
excessive amounts of ECM components, cytokines, and growth factors, including collagens, and tissue inhibitors of metalloproteinases. Specifically, the profibrotic cytokine TGF-β and its signaling pathway have been proposed to play important roles in the activation of SSc fibroblasts. This study will test the hypothesis that vibratory stimulation can reverse fibrotic pathological changes in matrix metabolism, restoring gene expression, collagen synthesis, and mechanical stiffness to levels not significantly different from normal skin fibroblasts.

2.2. Significance and Innovation

The United States government estimates that 45% of deaths in the United States can be attributed to fibrotic disorders [16,89]. Almost every major organ system is susceptible to fibrotic diseases; examples include pulmonary fibrosis, renal fibrosis, liver cirrhosis, arteriosclerosis, and scleroderma. Although considerable progress in the regression of fibrosis using TGF-β blocking strategies has been achieved over the past few years, the clinical application of these approaches is still limited because of the pleiotrophic activities of TGF-β and consequent side effects of systemic inhibition [126,180]. Therefore, the development of novel mechano-therapeutic approaches, with the opportunity for site-specific application and flexibility in handling and controlling doses, will address the limitations of current paradigms for antifibrotic therapies. Recently, the role of mechanical loading has drawn significant attention for its role in pathology and as a potential treatment [183]. Mechano-therapeutic approaches have potential benefits in specifically delivering mechanical stimuli to a target lesion, while conventional drug therapy systemically affects every tissue and organ. Currently,
mechanotherapies including microdeformational wound therapy (MDWT), shockwave therapy, soft tissue expansion, distraction osteogenesis, and surgical tension reduction are mainly focusing on treating and improving wound healing at the tissue level [183], however, the molecular mechanisms underlying the efficacy of these approaches remain to be thoroughly elucidated.

Chapter 3 will demonstrate that vibratory stimulation leads to the down-regulation of TGF-β signaling and inhibits collagen production and mechanical stiffening that are key phenotypic alterations observed in the tissues with fibrotic diseases.

In addition, development of mechano-therapeutic approaches has proven very challenging, particularly with regard to specificity, selectivity and timeliness [126,180]. Specific type, amplitude, duration, and frequency preferences could be carefully investigated to make a novel mechanobiology-based therapy more specific and effective and mechanical forces should also be applied with a finely tuned manner.

Chapter 4 will provide specific guidance that may be utilized in the development of a clinical mechanotherapy approach with maximum therapeutic efficacy.

Finally, chapter 5 will identify whether the vibratory stimulation could reverse the fibrotic phenotype on scleroderma-derived fibroblasts through (1) downregulation of TGF-β signaling, (2) attenuation of the gene expression of scleroderma phenotypic targets, and (3) reduction of the increases in collagen synthesis and matrix stiffness in SSc fibroblasts grown on polyurethane substrates.

In summary, fibrotic disease is commonly known as a progressive disorder with permanent changes in organs and tissues that results from over-expressed pro-fibrotic
cytokines and collagens. Although numerous types of antifibrotic therapies have been investigated, none of the current strategies effectively achieve restoration of the matrix composition and biomechanical properties of scarred tissue and organs. Therefore, these studies will offer a basis for the design of a novel class of mechanotherapy approach/device that may reverse the fibrotic phenotype in many fibrotic diseases and comprise the first steps towards the development of vibratory mechanotherapy as a clinical treatment for fibrotic disease.
3.1. Abstract

Mechanical stimuli play a central role in regulating extracellular matrix composition and organization during development, wound healing, and pathological fibrosis. The superficial layer of the lamina propria (SLLP) in the human vocal folds experiences a unique mechanical microenvironment of high frequency vibration during voice production. The identification of macrophages and myofibroblasts in the SLLP of healthy subjects suggests that the mechanical stresses imposed during routine speech result in repetitive microtrauma, which is generally repaired without permanent alterations in matrix composition or vocal quality. However, the mechanisms regulating fibroblast matrix remodeling in the SLLP have not been fully elucidated. Here we show that vibration downregulates transforming growth factor (TGF)-β signaling and inhibits the induction of collagen synthesis by fibrotic stimuli in human fibroblasts. Transcriptomic and protein-based assays revealed that vibration strongly induced the expression of the profibrotic cytokines TGFB1, TGFB2, connective tissue growth factor (CTGF), and endothelin-1 (EDN1). However, vibration also significantly affected the TGF-β signaling pathway, with a net effect of antagonizing signal transduction through reduced expression of TGF-β receptors and Smad signal transduction molecules and increased expression of SMAD7, ubiquitin ligases, and transcriptional repressors responsible for signaling inhibition. Vibration also inhibited cyclic strain- and TGF-β1-
mediated collagen accumulation and mechanical stiffening of fibroblast-seeded 3D, porous sponges, as well as reducing collagen content and mechanical stiffness of fibroblast-seeded sponges previously cultured under cyclic strain. These results suggest that vibration may offer the basis for development of a mechanomodulatory approach for the treatment of a wide range of fibrotic disorders.

3.2. Introduction

Tissues and organs contain a stromal component composed primarily of mesenchymally-derived cells and their secreted extracellular matrix (ECM) that provides structural and biomechanical support for parenchymal cells. The ECM is a dynamic structure that undergoes continuous remodeling through tightly regulated and coordinated processes of protein synthesis and proteolytic degradation [313]. Fibrosis results from pathological changes in the regulation of ECM remodeling, leading to excessive matrix accumulation that eventually compromises tissue function [16,86]. Although the molecular basis of fibrosis is not yet fully understood, cytokines and the mechanical microenvironment are recognized as important regulators of ECM remodeling [314,315]. Transforming growth factor (TGF)-β1, TGF-β2, connective tissue growth factor (CTGF), and endothelin 1 (EDN1) are profibrotic cytokines that stimulate ECM synthesis and are chronically over-expressed in a variety of fibrotic diseases [316]. Mechanical factors, including both changes in matrix stiffness and application of external loads, can also influence matrix remodeling [317]. While many antifibrotic strategies seek to reduce cytokine expression levels and signaling activity [318], relatively few studies have
investigated the potential of mechanical factors for therapeutic modulation of matrix remodeling.

The vocal fold lamina propria is a laryngeal connective tissue that is unique in terms of its matrix composition and biomechanical function. Residing between a squamous epithelium and the thyroaretenoid muscle, the lamina propria develops from a relatively homogeneous tissue in newborns to a highly organized, layered anatomical structure in adolescents [292,319]. The superficial layer of the lamina propria (SLLP) is composed of reticular fibers, amorphous forms of elastin, hyaluronic acid, and proteoglycans, while the intermediate and deep layers are enriched in mature elastic and collagen fibers, respectively [280,282,320]. During phonation, the SLLP vibrates at frequencies ranging from 100-300 Hz and amplitudes up to 1 mm, while the vocal ligament, comprising the intermediate and deep layers of the lamina propria, undergoes cyclic strain at lower (1-10 Hz) frequency [288]. Histological analysis has shown that macrophages and myofibroblasts are consistently present in the vocal folds of healthy subjects and concentrated in the SLLP [284,293,299]. On the basis of these observations, it has been proposed that normal phonation results in repetitive microtrauma that is routinely repaired without permanent changes in matrix composition or adverse effects on vocal quality (18). However, the underlying mechanisms responsible for this healing capacity remain unknown.

One of the fundamental principles of mechanobiology that has been demonstrated across a wide variety of cell types is that physiologically-relevant mechanical loading stimulates tissue-specific matrix gene expression [321–323]. The limited amount of
mature fibrous ECM in the native SLLP suggests that exposure to high frequency vibration may inhibit fibroblast expression and synthesis of fibrous matrix and offer a basis for novel antifibrotic therapies. Recently, mechanical bioreactors have been used to investigate vibration-induced changes in matrix-related gene expression in vitro. Despite substantial variations in cell type, substrate, bioreactor design, and vibration protocol, many studies have reported increased expression of genes consistent with the native tissue physiology and potentially providing resistance to scarring, including up-regulation of hyaluronic acid synthases, proteoglycans, and matrix metalloproteinase 1 (MMP1) and no significant change or down-regulation of type I collagen [324–329]. One limitation of these studies is that they have primarily relied upon RT-PCR analysis of a limited number of targets and revealed little about the signaling pathways responsible for the effects observed.

Here, we have performed a comprehensive analysis of the transcriptional response of human dermal fibroblasts to high frequency vibration. While exposure to vibration significantly increases expression of profibrotic cytokines, it simultaneously decreases expression of fibrous collagens and inhibits the TGF-β signaling pathway. Using cyclic strain and exogenous TGF-β1 as fibrotic models, we show that vibration counteracts collagen accumulation and mechanical stiffening observed in a wide range of fibrotic diseases.

3.3. Materials and Methods

3.3.1. Vibratory bioreactor
The vibratory bioreactor module consisted of a modified T-75 flask as a culture module and a bioreactor platform equipped with an electromagnetic voice coil actuator (BEI Kimco, Vista, CA) and a stepper motor (PKK 244-02BA, Oriental Motor, Charlotte, NC) constructed as previously described [322]. The voice coil actuator was connected to a horizontal plastic tube holding 4 plastic rods that passed through flexible gaskets into the interior of the flask upon which one end of each of 4 samples was mounted. The voice coil actuator was driven by a sine wave from a frequency generator (4040A, B&K Precision, Yorba Linda, CA) processed through a 10W amplifier (CK003, Cana Kit, North Vancouver, Canada) and connected through a reed switch to the collector terminal (+5 V from a PC) of a NPN transistor, the base terminal of which was connected to the output from a custom LabVIEW program (National Instruments, Austin, TX) running on a PC. On the other side of each bioreactor platform, a stepper motor was mounted and connected by a pulley to a lead screw controlling the movement of an actuation bar mounted on a linear slide assembly with 4 attached sample holding bars that passed into the flask module. The stepper motor was controlled through stepper motor drivers (G201, Geckodrive, Tustin, CA) and a 4-axis motion control card (DMC-18x2, Galil, Rocklin, CA). The vibration and cyclic strain regimes were controlled by a custom LabVIEW program. Porous, 3D sponges were fabricated from the elastomeric polyurethane (Tecoflex® SG-80A, Lubrizol Corporation, Wickliffe, OH) by a phase separation/precipitation method as previously described [325].

3.3.2. Substrate strain measurement
To calculate the actual substrate strains occurring within polyurethane sponges during vibration, eight small dots of 0.7% Sudan Black B in two parallel rows were placed on the surface of samples (25 mm in length x 5 mm in width x 2 mm in height) as fiducial markers. During vibration, the oscillatory displacement of the substrate was recorded using a Sony Handycam Camcorder (HDR-CX360) under a digital stroboscope at a 1 Hz frequency offset. Images corresponding to both positions of neutral and maximum sample displacement were extracted and substrate strains were determined by measuring the initial and final separation distances between markers oriented parallel and perpendicular to the direction of vibratory oscillation using Photoshop.

3.3.3. Cell culture

Adult normal human dermal fibroblasts (NHDFs; Lonza, Allendale, NJ) were cultured in tissue culture flasks (Greiner Bio-one, Monroe, NC) with Dulbecco’s Modification of Eagle’s Medium/Ham’s F-12 50/50 Mixture with L-glutamine (DMEM/F12; Mediatech, Herndon, VA) supplemented with 10% v/v bovine growth serum (BGS; Hyclone, Logan, UT) and 1% penicillin-streptomycin (Mediatech). Adult human aortic adventitial fibroblasts (AoAF; Lonza) and adult normal human lung fibroblasts (NHLF; kindly donated by Dr. Carol Feghali-Bostwick, Medical University of South Carolina) were cultured in tissue culture flasks with Dulbecco's Modification of Eagle's Medium (DMEM; Mediatech) supplemented with 10% v/v fetal bovine serum (FBS; Hyclone) and 1% penicillin-streptomycin. Medium was changed every 3 days and cells were passaged at a 1:3 ratio for expansion.

3.3.4. Bioreactor culture for microarray, qRT-PCR, and protein analysis
1.2 x 10^6 NHDF between fourth and sixth passages were seeded on each 3D, porous polyurethane sponge (25 mm in length x 5 mm in width x 2 mm in height) mounted on a polyester frame and cultured in a 100 mm Petri dish containing DMEM/F12 with 10% BGS and 1% penicillin-streptomycin for 4 days under static conditions. The experimental group was subjected to a one-time 20% axial strain to ensure the samples were all under approximately equal initial tension followed by vibration at 100 Hz frequency, 3.8 Vrms, 1.28 ± 0.07 mm amplitude (“1X Strain + Vibration”). Two control groups were used, one loaded in bioreactors and exposed to the initial tensioning strain only (“1X Strain”) and another maintained under completely static conditions (“Static”).

3.3.5. Microarray analysis

After 6 hours culture under vibratory or control conditions, total RNA was immediately isolated by Trizol extraction followed by purification using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer’s protocols. Total RNA samples were evaluated for quality using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Total RNA samples were converted to fragmented, biotin-labeled cRNA using the GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA) and then hybridized to Human PrimeView microarrays (Affymetrix) according to manufacturer recommendations. Probe set expression values were extracted and normalized by RMA algorithm using Expression Console software (Affymetrix). To identify gene differentially expressed between the 1X Strain + Vibration samples and 1X Strain samples, we employed a statistical test (Student’s t-test, unpaired, 2-tailed, not
assuming equal variance) as well as a fold change threshold. The combination of these two tests allows detection of statistically significant differences that had magnitude changes that could be validated by other conventional mRNA measurement methods. Comparative gene expression was conducted with dChip software [330]. Student’s t-test and fold change thresholds were evaluated through empirical testing to find values that satisfied two outcomes: 1) estimated false discovery for the group of genes identified as differentially expressed would be \( \leq 5\% \); 2) the group of genes identified would be of sufficient size to permit functional enrichment analysis. Criteria that satisfied these conditions were absolute fold change \( \geq 2 \) and \( P < 0.05 \). The false discovery rate estimated for these criteria, based on iterative permuted comparisons involving randomized sample groupings, was 0.0%. ECM component genes were collected based on the Gene Ontology tag extracellular matrix component (GO:0044420); TGF-\( \beta \) signaling pathway genes were collected based on KEGG pathway identification (hsa04350) and via manual assignment. To identify genes differentially expressed within the ECM component group and TGF-\( \beta \) signaling pathway group, we chose to apply only a statistical test (Student’s t-test, unpaired, 2-tailed, not assuming equal variance). This was chosen because group sizes were relatively small and thus multiple testing errors would be expected to be less prevalent that in comparisons involving total microarray content. Additionally, this would be less stringent and therefore more permissive for detecting genes within the groups that were affected by vibration. Evaluation of differential expression analyses conducted at a threshold of \( P < 0.05 \) revealed false discovery rates approximated 4.5% for differentially expressed ECM component genes and 6.2% for TGF-\( \beta \) signaling pathway genes. Gene
ontology enrichment and KEGG Pathway enrichment analyses were conducted with the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 tool [331,332]; enrichment scores were considered significant at $P < 0.05$. Raw data (CEL files) and normalized expression data produced through this microarray study are deposited in the NCBI Gene Expression Omnibus (accession GSE68386).

Table 3.1. Primer sequences used in Real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse Primer (5’–3’)</th>
<th>GeneBank no.</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1</td>
<td>TCCACGGGAGAAGCAGTCCG</td>
<td>TCCACGGGAGAAGCAGTCCG</td>
<td>NM_000660.5</td>
<td>137</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>AACGCAAACTTCTGTCGAG</td>
<td>AACGCAAACTTCTGTCGAG</td>
<td>NM_003238.3</td>
<td>117</td>
</tr>
<tr>
<td>CTGF</td>
<td>TCCATTTCAACCACACCCAGA</td>
<td>TCCATTTCAACCACACCCAGA</td>
<td>NM_001901.2</td>
<td>141</td>
</tr>
<tr>
<td>EDN1</td>
<td>CAGAAAAGTGTTGAGAAGGTT</td>
<td>CAGAAAAGTGTTGAGAAGGTT</td>
<td>NM_00168319.1</td>
<td>123</td>
</tr>
<tr>
<td>COL1A1</td>
<td>TCCTTCGTCTCCTGTTGCTT</td>
<td>TCCTTCGTCTCCTGTTGCTT</td>
<td>NM_000088.3</td>
<td>142</td>
</tr>
<tr>
<td>COL3A1</td>
<td>CACAACCCACTCTGAAATG</td>
<td>CACAACCCACTCTGAAATG</td>
<td>NM_000090.3</td>
<td>117</td>
</tr>
<tr>
<td>COL5A2</td>
<td>AGGAGCTCTGACAGAAGAGC</td>
<td>AGGAGCTCTGACAGAAGAGC</td>
<td>NM_000393.3</td>
<td>146</td>
</tr>
<tr>
<td>TGFBR1</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>NM_00130621.0</td>
<td>148</td>
</tr>
<tr>
<td>TGFBR2</td>
<td>TGCTCAGATACTGAGGATG</td>
<td>TGCTCAGATACTGAGGATG</td>
<td>NM_00124847.2</td>
<td>132</td>
</tr>
<tr>
<td>TGFBR3</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>NM_00119568.3</td>
<td>131</td>
</tr>
<tr>
<td>SMAD3</td>
<td>AGGATCTCTGTCCTCGAC</td>
<td>AGGATCTCTGTCCTCGAC</td>
<td>NM_001145103.1</td>
<td>123</td>
</tr>
<tr>
<td>SMAD7</td>
<td>TGAGGAGCTCTGAGGATG</td>
<td>TGAGGAGCTCTGAGGATG</td>
<td>NM_00119082.1</td>
<td>129</td>
</tr>
<tr>
<td>SMURF1</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>NM_00119984.7</td>
<td>134</td>
</tr>
<tr>
<td>SMURF2</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>NM_001273.9</td>
<td>139</td>
</tr>
<tr>
<td>SIK1</td>
<td>AAGGATCTCTGTCCTCGAC</td>
<td>AAGGATCTCTGTCCTCGAC</td>
<td>NM_173354.3</td>
<td>108</td>
</tr>
<tr>
<td>B2M</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>TGGCATCAGCAGGATAAGG</td>
<td>NM_004048.2</td>
<td>137</td>
</tr>
</tbody>
</table>

*All primer sequences were designed based on gene sequence obtained from the respective GenBank numbers using Primer–BLAST (http://www.ncbi.nlm.nih.gov/tools/primer–blast/). Abbreviation: TGFβ, transforming growth factor-β; CTGF, connective tissue growth factor; EDN1, endothelin 1; COL, collagen; TGFBR, TGF-β receptor; SMURF, smad–specific E3 ubiquitin protein ligase; SIK1, salt-inducible kinase 1; SKIL, SKI-like proto-oncogene; B2M, beta-2–microglobulin.

3.3.6. qRT-PCR

After 0.25, 1, 3, and 7 day culture under experimental/control conditions described above, total RNA was isolated by combining TRIzol reagent (Invitrogen) and RNeasy Mini Kit (Qiagen) as described above. Two step real time RT-PCR was performed using Retroscript kit (Ambion, Austin, TX), Quantitect SYBR green PCR kit (Qiagen), and target-specific primers (Table 3.1) using a Rotor-Gene 3000 Real-Time
Thermal Cycler (Qiagen). Relative gene expression levels were calculated using $2^{-\Delta\Delta Ct}$ method with beta-2-microglobulin (B2M) as an internal standard [333]. For the 0.25 day time point, $n = 8$ total samples were analyzed ($n = 4$ from the samples used for microarray analysis and $n = 4$ from an independent replicate).

3.3.7. ELISA assay

Bioreactor flask modules were modified with plastic inserts to create an individual compartment for each sample. NHDF seeded in 3D, porous polyurethane sponges were cultured under experimental/control conditions described above and culture medium samples collected at 1, 3, and 7 days. The concentration of connective tissue growth factor (CTGF) secreted into the culture medium was determined using a sandwich ELISA kit (Antigenix America, Huntington Station, NY). Ninety-six well high protein binding ELISA plates (Greiner Bio-one) were coated with 2.0 mg/ml anti-CTGF capture antibody overnight at room temperature. Plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with a 1% BSA solution for 2 h at room temperature. One hundred microliter aliquots of CTGF standards or samples were added to wells and incubated for 2 h at room temperature. After washing, 100 ml of 0.1 mg/ml biotin labeled anti-CTGF detection antibody was added to wells and incubated at room temperature for 2 h. Plates were again washed and 100 ml of 0.15 mg/ml peroxidase-conjugated streptavidin was added to each well, incubated at room temperature for 30 min, and washed. Following the addition of 100 ml aliquots of fluorogenic substrate, the absorbance was measured at 390 nm using a microplate reader (Biotek Instruments, Winooski, VT). The concentration of TGF-β1 (RayBiotech, Norcross, GA), TGF-β2
(R&D Systems, Minneapolis, MN), and endothelin-1 (R&D Systems) were also determined using a sandwich ELISA process according to manufacturer’s instructions. For analysis of TGF-β1 and TGF-β2, media samples were activated for 10 min by acidification using 1 N HCl followed by neutralization using 1.2 N NaOH/0.5 M HEPES.

3.3.8. Western blot analysis; TGF-β signaling

NHDF seeded in 3D, porous polyurethane sponges were cultured under experimental/control conditions as described above. At 1, 3, and 7 days, sponges were collected, washed three times with PBS, and lysed using RIPA lysis buffer (150 mM Tris base (pH 7.6), 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100) supplemented with proteinase inhibitor cocktail (cOmplete™, Roche, Mannheim, Germany) and phosphatase inhibitor cocktail (PhosSTOP™, Roche). Total protein concentration in the lysates was measured by BCA assay (Pierce, Rockford, IL). Samples (20 mg) were electrophoretically separated on SDS–polyacrylamide gel (10-17%) and transferred to a PVDF membrane (Millipore, Bedford, MA, U.S.A.). Membranes were soaked in 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) for 2 h (except for the detection of phosphorylated proteins which was incubated in 5% horse serum in TTBS overnight at 4 °C) to block nonspecific binding. Membranes were incubated overnight at 4 °C with primary antibodies against SMAD3 (04-1035, EMD Millipore, Chicago, IL), phospho-SMAD3 (07-1389, EMD Millipore), SMAD7 (ST1625, Calbiochem), transforming growth factor b receptor 1 (TGFBR1) (06-1086, EMD Millipore), TGFBR2 (06-227, EMD Millipore), TGFBR3 (MABC710, EMD Millipore), salt-induced kinase 1 (SIK1) (ABE799, EMD Millipore), SKIL/SnoN (07-077, EMD
Millipore), or beta-2-microglobulin (B2M) (ab75853, Abcam, Cambridge, MA), washed three times with TTBS, and then incubated again with appropriate secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) for 50 min at room temperature. Blots were developed using the Clarity™ Western ECL substrate (Bio-Rad) following the manufacturer’s protocols. Luminescence was captured using a GS-690 imaging system (Bio-Rad) and densitometric analysis was performed with ImageJ software. Quantitative data normalized to B2M densitometry levels are expressed as mean ± standard deviation (SD) (Fig. 3.5).

3.3.9. In vitro fibrotic models

In order to model fibrotic conditions by stimulating collagen synthesis and mechanical stiffening, NHDF, AoAF, and NHLF were seeded in 3D, porous polyurethane sponges (1.2 x 10⁶ cells/sponge) and initially cultured for 4 days under static conditions as described above. Cells were exposed to pro-fibrotic conditions (exogenous TGF-β1 supplementation or cyclic strain) either with or without simultaneous application of vibratory stimulation. Once samples were transferred to bioreactors, all groups including controls were supplemented with 1 mM L-ascorbic acid 2-phosphate (Sigma) to support functional collagen deposition. For the TGF-β1 model, NHDF-seeded sponges were subjected to a one-time 20% static strain and then cultured under static or vibratory loading (100 Hz, 1.28 ± 0.07 mm amplitude) with and without 10 ng/ml recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ) for 6 h/day. For the cyclic strain model, NHDF-, AoAF-, and NHLF-seeded sponges were subjected to a one time, 15% static strain (3 mm strain amplitude) for initial tensioning, followed by 5% axial
cyclic strain (1 mm strain amplitude, 0.25 Hz) alone or in combination with vibration under tension (100 Hz, 1.28 ± 0.07 mm amplitude, 3.05 s on/0.95 s off) for 8 h/day. The vibratory ‘off’ period was required for stretch and relaxation during each strain cycle, while the 8 hour total time maintained 6 hours total active vibration, consistent with all previous studies. For the final set of studies to test whether vibratory stimulation could promote the remodeling of the newly synthesized matrix, NHDF-seeded 3D, porous polyurethane sponges were cultured under cyclic strain (5%, 0.25 Hz) for one week and then subjected to a one-time 15% static strain followed by either static or vibratory (100 Hz, 1.28 ± 0.07 mm amplitude for 6 h/day) culture for an additional week.

3.3.10. Hydroxyproline assay

Collagen accumulation was quantified by the hydroxyproline assay as previously described [334]. Briefly, polyurethane sponges were digested in 1 ml of 12 N HCl and then hydrolyzed at 98 °C for 3 hours. After centrifugation, 20 µl aliquots of standards (4-hydroxyproline, 0–400 µg/ml) and samples were transferred to a 96 well plate, then dried in a vacuum oven overnight. Samples were mixed with 100 µl of chloramine T solution [141 mg/ml chloramine-T, 1 ml n-propanol and 8 ml OH-Pro buffer (1.25 g citric acid, 3 g sodium acetate, 0.6 g NaOH, dissolved in 30 ml distilled water containing 0.3 ml glacial acetic acid and 7.5 ml n-propanol, pH 6)]. Samples were mixed with 100 µl p-dimethyl amino benzaldehyde solution (0.75 g p-dimethyl amino benzaldehyde in 3 ml n-propanol and 1.3 ml 70% v/v perchloric acid), incubated for 20 min at room temperature, and heated to 60 °C for 15 min. The absorbance was measured at 558 nm. Total collagen content was calculated based on the estimation that hydroxyproline comprises about
13.5% of the total collagen. Total collagen values were normalized to total DNA values measured by using a PicoGreen assay (Molecular Probes, Eugene, OR). All chemicals and solvents used in this study were purchased from Sigma-Aldrich and of analytical reagent grade.

3.3.11. Tensile testing

NHDF-seeded samples were washed with PBS and cut free from the plastic frames. The width and thickness of samples were measured using digital calipers. Samples were mounted using rough-surfaced aluminum grips and set to a 5 mm separation distance. The samples were strained to failure at 1 mm/s using an MTS Synerge 100 (MTS Systems Corporation, Eden Prairie, MN) and data analyzed using Testworks 4 software (MTS Systems Corporation). Construct elastic modulus was calculated by a peak slope method, generally corresponding to the 30% to 60% strain region as previously described [231].

3.3.12. Statistical analysis

Data analysis was done by using GraphPad Prism software (GraphPad, San Diego, CA). Significant differences between groups were determined with one-way analysis of variance (ANOVA) followed by Tukey multiple pairwise comparison tests. \( P < 0.001 \) was accepted as statistically significant. All quantitative data are presented as mean ± standard deviation (SD).

3.4. Results

3.4.1. Vibration elicits differential gene expression in human dermal fibroblasts
To examine the transcriptional response caused by vibratory mechanical stimulation, normal adult human dermal fibroblasts (NHDF) were cultured in 3D, porous polyurethane sponges and loaded in a vibratory bioreactor as previously described [324]. The experimental group was subjected to a one-time 20% axial strain to ensure the samples were all under approximately equal initial tension followed by vibration at 100 Hz and 1.28 ± 0.07 mm amplitude (“1X Strain + Vibration”). Measurements of the displacement of fiduciary markers during vibration indicated that this mechanical stimulus produced corresponding substrate strains of 0.25 ± 0.09% and 1.65 ± 0.23% parallel and perpendicular to the axis of oscillatory motion, respectively. Two control groups were used, one loaded in bioreactors and exposed to the initial tensioning strain only (“1X Strain”) and another maintained under completely static conditions (“Static”). After 6 h culture, total RNA samples were collected and subjected to microarray analysis. Findings indicated that vibratory stimulation caused significant changes in gene expression (P < 0.05 and fold change > 2), with 778 genes up-regulated and 557 significantly down-regulated in 1X Strain + Vibration samples compared to 1X Strain samples (Fig. 3.1A). Expression patterns revealed that many of these genes were influenced by vibration in a manner that was independent of axial strain, i.e., these genes showed no overt response to 1X Strain relative to the Static condition (Fig. 3.1A, genes highlighted by black bars). However, other genes responded to vibration in a manner that resembled either an augmentation or a reversal of the response to 1X Strain relative to the Static condition (Fig. 3.1A, genes highlighted by white bars). Although the static condition was included as a baseline reference, all subsequent analyses focused on
differences between the 1X Strain + Vibration and 1X Strain groups to identify changes solely attributable to the effect of vibration.

Figure 3.1. Vibration stimulates differential gene expression including ECM and TGF-β signaling components. (A) Global analysis of all transcripts represented on the microarrays detected 1335 differentially expressed genes \((P < 0.05\) and fold change \(> 2\)) affected by 1X Strain + Vibration compared to 1X Strain groups. Analyses of ECM (B) and TGF-β signaling pathway components (C) identified 110 (27.2%) and 92 (36.9%) genes significantly affected \((P < 0.05)\) by 1X Strain + Vibration relative to 1X Strain, respectively. The black bars on the right side of heat maps represent gene clusters that were solely influenced by vibration, while the white bars represent genes that responded to vibration as well as strain. \(n = 3\) independent samples per group.
Biological pathways (KEGG, Kyoto Encyclopedia of Genes and Genomes) and biological processes (GO, Gene Ontology) that were significantly affected by 1X Strain + Vibration relative to 1X Strain are summarized in Table 3.2 and 3.3, respectively. Vibration responsive genes were enriched for a variety of growth factor signaling pathways, notably including the TGF-β pathway (Table 3.2). Consistent with the broad changes in gene expression elicited by vibration, RNA polymerase II dependent transcription was one of the most significantly enriched biological process categories. Also of note were multiple processes relating to tissue repair including regulation of cell proliferation, regulation of cell migration, angiogenesis, and response to wounding (Table 3.3).

Table 3.2. Biological pathways significantly enriched in NHDFs subjected to 1X Strain + Vibration relative to 1X Strain.

<table>
<thead>
<tr>
<th>KEGG Pathway*</th>
<th>ID†</th>
<th>Count‡</th>
<th>P value§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathways in cancer</td>
<td>hsa05200</td>
<td>39</td>
<td>8.7E-07</td>
</tr>
<tr>
<td>TGF-β signaling pathway</td>
<td>hsa04350</td>
<td>15</td>
<td>1.0E-04</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>hsa05210</td>
<td>13</td>
<td>1.0E-03</td>
</tr>
<tr>
<td>ErbB signaling pathway</td>
<td>hsa04012</td>
<td>13</td>
<td>1.3E-03</td>
</tr>
<tr>
<td>Cytokine–cytokine receptor interaction</td>
<td>hsa04060</td>
<td>24</td>
<td>6.6E-03</td>
</tr>
<tr>
<td>MAPK signaling pathway</td>
<td>hsa04010</td>
<td>24</td>
<td>8.2E-03</td>
</tr>
<tr>
<td>Wnt signaling pathway</td>
<td>hsa04310</td>
<td>16</td>
<td>9.1E-03</td>
</tr>
<tr>
<td>Jak–STAT signaling pathway</td>
<td>hsa04630</td>
<td>16</td>
<td>1.2E-02</td>
</tr>
<tr>
<td>VEGF signaling pathway</td>
<td>hsa04370</td>
<td>10</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>Basal cell carcinoma</td>
<td>hsa05217</td>
<td>8</td>
<td>2.0E-02</td>
</tr>
</tbody>
</table>

*Biological pathways defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. †KEGG pathway database identification number (ID). ‡Number of genes in the pathway that were differentially expressed. §p value enrichment score calculated by the Database for Annotation, Visualization and Integrated Discovery (DAVID).
Table 3.3. Biological processes significantly enriched in NHDFs subjected to 1X Strain + Vibration relative to 1X Strain.

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>ID</th>
<th>Count</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of cell proliferation</td>
<td>GO:0042127</td>
<td>93</td>
<td>3.1E-16</td>
</tr>
<tr>
<td>Regulation of transcription from RNA polymerase II promoter</td>
<td>GO:0006357</td>
<td>84</td>
<td>4.0E-14</td>
</tr>
<tr>
<td>Skeletal system development</td>
<td>GO:001501</td>
<td>49</td>
<td>8.5E-13</td>
</tr>
<tr>
<td>Regulation of RNA metabolic process</td>
<td>GO:0051252</td>
<td>150</td>
<td>2.8E-12</td>
</tr>
<tr>
<td>Regulation of cell motion</td>
<td>GO:0051270</td>
<td>36</td>
<td>5.5E-12</td>
</tr>
<tr>
<td>Enzyme linked receptor protein signaling pathway</td>
<td>GO:0007167</td>
<td>49</td>
<td>1.1E-11</td>
</tr>
<tr>
<td>Positive regulation of cellular biosynthetic process</td>
<td>GO:0031328</td>
<td>74</td>
<td>4.5E-11</td>
</tr>
<tr>
<td>Regulation of cell migration</td>
<td>GO:0030334</td>
<td>31</td>
<td>3.2E-10</td>
</tr>
<tr>
<td>Positive regulation of nitrogen compound metabolic process</td>
<td>GO:0051173</td>
<td>69</td>
<td>3.3E-10</td>
</tr>
<tr>
<td>Vasculature development</td>
<td>GO:0001944</td>
<td>37</td>
<td>2.5E-09</td>
</tr>
<tr>
<td>Negative regulation of cell differentiation</td>
<td>GO:0045596</td>
<td>33</td>
<td>8.8E-09</td>
</tr>
<tr>
<td>Embryonic morphogenesis</td>
<td>GO:0048598</td>
<td>39</td>
<td>5.5E-08</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>GO:0001525</td>
<td>25</td>
<td>1.1E-07</td>
</tr>
<tr>
<td>Positive regulation of developmental process</td>
<td>GO:0051094</td>
<td>36</td>
<td>1.2E-07</td>
</tr>
<tr>
<td>Response to wounding</td>
<td>GO:0009611</td>
<td>54</td>
<td>2.0E-07</td>
</tr>
<tr>
<td>Skeletal system morphogenesis</td>
<td>GO:0048705</td>
<td>21</td>
<td>2.6E-07</td>
</tr>
<tr>
<td>Regulation of programmed cell death</td>
<td>GO:0043067</td>
<td>72</td>
<td>3.3E-07</td>
</tr>
<tr>
<td>Epithelium development</td>
<td>GO:0060429</td>
<td>31</td>
<td>3.4E-07</td>
</tr>
<tr>
<td>Transmembrane receptor protein tyrosine kinase signaling pathway</td>
<td>GO:0007169</td>
<td>30</td>
<td>8.2E-07</td>
</tr>
<tr>
<td>Regulation of apoptosis</td>
<td>GO:0042981</td>
<td>70</td>
<td>9.9E-07</td>
</tr>
</tbody>
</table>

*Biological processes defined by the Gene Ontology database. 1Gene Ontology database identification number (ID). 2Number of differentially expressed genes that are annotated with the biological process. 3P value enrichment score calculated by the Database for Annotation, Visualization and Integrated Discovery (DAVID).

Given the established connections between mechanical stimulation and ECM remodeling and the fact that the TGF-β pathway is prominently involved in matrix regulation, we performed new analyses of the microarray data that focused on genes encoding ECM components and TGF-β signaling molecules. Of the 405 ECM component genes represented on the microarray, 110 (27.2%) were significantly affected by 1X Strain + Vibration relative to 1X Strain (P < 0.05; Fig. 3.1B), confirming that vibration exerts a substantial influence on ECM gene expression. Similarly, 92 of 249 of genes related to the TGF-β signaling pathway (36.9%) were significantly affected by 1X Strain
Vibration compared to the 1X Strain group (P < 0.05), including those involved in both BMP/activin and TGF-β/Smad signaling (Fig. 3.1C).

Figure 3.2. Vibration significantly affects mRNA expression levels of genes associated with matrix remodeling. *Fold change (FC) values are displayed for selected target genes encoding cytokines, fibrous collagens and regulators of matrix turnover, and components of the TGF-β signaling pathway exhibiting significant differences (P < 0.05) in mRNA expression levels in response to 1X Strain + Vibration relative to 1X Strain group (n = 3 independent samples per target). Target abbreviations are color-coded to identify genes involved in stimulating (red) and inhibiting (green) fibrous matrix deposition and TGF-β signal transduction. †P value enrichment score calculated by the Database for Annotation, Visualization and Integrated Discovery (DAVID). ‡Check marks identify targets displaying a change in expression considered indicative of an antifibrotic effect (AF) defined as increased expression of targets that inhibit matrix deposition and TGF-β signal transduction and decreased expression of targets that stimulate matrix deposition and TGF-β signal transduction.

Figure 3.2 summarizes genes related to matrix remodeling (cytokines, ECMs, and TGF-β/Smad signaling pathway components) showing significant changes (P < 0.05) in expression levels in response to vibration. Interestingly, profibrotic cytokines (TGFB1, TGFB2, CTGF, and EDN1) were up-regulated, while fibrous collagens were down-regulated. Among numerous components of the TGF-β signaling pathway showing
differential expression, the majority of changes favored an antifibrotic effect defined as a down-regulation of genes that promote TGF-β signal transduction and an up-regulation of those involved in signaling inhibition/termination. Overall, these results suggest that while vibratory simulation increases the expression of profibrotic cytokines, it simultaneously modulates signaling pathways to inhibit their activity.

3.4.2. Vibration increases expression of profibrotic cytokines, but not fibrous collagens

qRT-PCR and ELISA assays were performed to validate the microarray results for cytokine and collagen expression at the 6 hour (0.25 day) time point and investigate if these changes were sustained when vibration was applied for 6 h/day for 1, 3 and 7 days. With respect to 1X Strain controls, qRT-PCR results confirmed that vibration resulted in significant increases in expression of profibrotic cytokines TGFB1, TGFB2, CTGF, and EDN1 at all time points with the exceptions of TGFB1 at 0.25 days and EDN1 at 7 days (Fig. 3.3A). mRNA expression levels of collagens COL1A1, COL3A1, and COL5A2 in fibroblasts exposed to vibration were either significantly lower or not significantly different from 1X Strain controls at all time points (Fig. 3.3B). In contrast, expression of the collagenase MMP1 was significantly increased at Days 0.25, 3, and 7. Because increases in profibrotic cytokine gene expression did not result in increased collagen expression as would normally be expected, ELISA assays were performed to confirm that increases in cytokine mRNA resulted in increased protein expression. Consistent with the mRNA results, protein levels of TGFB1, TGFB2, CTGF and EDN1 were significantly higher in vibrated samples relative to 1X Strain controls at 1 and 3 days and for TGFB1 at 7 days, although protein levels for all cytokines except TGFB1 significantly decreased.
between day 1 and 7 (Fig. 3.3C). Collectively, these results suggest that vibration interferes with the induction of collagen matrix expression that normally results from increased expression of profibrotic cytokines.

Figure 3.3. Vibration increases expression of profibrotic cytokines, but not fibrous collagens. Relative mRNA expression levels of profibrotic cytokines (A) and fibrous collagens (B). All data were normalized to static control at 0.25 days. $n = 8$ for 0.25 day time point and $n = 4$ for 1, 3, and 7 day time points. (C) Soluble protein concentrations of profibrotic cytokines released in the culture medium measured by ELISA assay. $n = 4$ samples per group. *(P < 0.001) compared with 1X Strain group at the same time point, †(P < 0.001) compared with 1X Strain + Vibration group at 0.25 day time point. All bar graphs, mean ± SD.
3.4.3. Vibration negatively regulates the TGF-β signaling pathway

To investigate the possibility that down-regulation of TGF-β signaling observed in vibrated samples might block collagen induction despite increased expression of profibrotic cytokines, we further investigated the expression levels of TGF-β receptors, Smad signaling intermediaries, ubiquitin ligases involved in receptor degradation (Smad specific E3 ubiquitin protein ligase (SMURF)1, SMURF2, and salt-inducible kinase 1 (SIK1)), and the nuclear transcriptional regulator SKI-like proto-oncogene (SKIL) by qRT-PCR and Western blotting after 0.25, 1, 3, and 7 days of vibratory culture. Among targets that facilitate TGF-β signal transduction, mRNA expression levels of TGF-β receptor type II (TGFBR2), TGFBR3 and SMAD3 were significantly reduced in vibrated samples relative to 1X Strain controls at all time points with the exception of TGFBR2 at 3 days (Fig. 3.4A).

In contrast, mRNA expression levels of targets involved in signaling inhibition (SMAD7, SMURF1, SIK1, and SKIL) were significantly up-regulated at all time points and SMURF2 at day 3 and 7 relative to 1X Strain controls (Fig. 3.4B). Western blot analysis also confirmed down-regulation of signal transduction molecules and up-regulation of signaling inhibitors (Fig. 3.4C, quantification of densitometry provided in Fig. 3.5). In general, changes in expression observed at day 1 became more pronounced at 3 and 7 days, most notably with expression of all three TGF-β receptors dropping to almost undetectable levels by day 7. These results suggest that inhibition of TGF-β signaling through a combination of reduced expression of receptors/signal transduction molecules and induction of negative feedback/inhibitory pathways is one mechanism by
which vibration inhibits induction of collagen synthesis despite elevated expression of profibrotic cytokines.

Figure 3.4. Vibration inhibits TGF-β signaling through down-regulation of targets involved in signal reception/transduction and up-regulation of signaling inhibitors. Relative mRNA expression levels of targets involved in TGF-β signal transduction (A) and inhibition (B). All data were normalized to static control at 0.25 days. All bar graphs, mean ± SD. n = 8 for 0.25 h time point and n = 4 for 1, 3, and 7 day time points. *(P < 0.001). (C) Western blot analysis of TGFBR1, TGFBR2, TGFBR3, SMAD3, phosphorylated Smad3 (pSMAD3), SMAD7, SIK1, SKIL and beta-2-microglobulin (B2M) used as a control. n = 4 samples per group. *(P < 0.001) compared with 1X Strain group at the same time point, †(P < 0.001) compared with 1X Strain + Vibration group at 0.25 day time point.
Figure 3.5. Densitometric analysis of western blots. Protein expression (TGFBR1, TGFBR2, TGFBR3, SMAD3, phosphorylated Smad3 (pSMAD3), SMAD7, SIK1, and SKIL) was normalized based on the densitometry of beta-2-microglobulin (B2M) that was used as an endogenous control. \( n = 3 \) independent experiments. *\((P < 0.001)\) compared with 1X Strain group at the same time point, †\((P < 0.001)\) compared with 1X Strain + Vibration group at 1 day time point. All bar graphs, mean ± SD.
3.4.4. Vibration blocks collagen accumulation and mechanical stiffening in fibrotic models \textit{in vitro}

In order to demonstrate that the responses to vibration observed at the molecular level may have functional significance in the context of wound healing and fibrotic disease, the effects of vibration on fibroblasts were evaluated using two \textit{in vitro} models well-established to stimulate collagen synthesis and mechanical stiffening. In the first model, NHDF-seeded sponges were cultured under static or vibratory conditions in the presence of TGF-β1. Relative to untreated static controls, TGF-β1 stimulated significant increases in collagen accumulation and stiffness in static samples. However, when samples were cultured under vibratory conditions in the presence of TGF-β1, no significant changes in either outcome were observed (Fig. 3.6A).

In the second model, cell-seeded samples were subjected to cyclic strain alone or in combination with vibration. In order to test the broader applicability of vibratory stimulation, these studies were performed with NHDF, human aortic adventitial fibroblasts (AoAF), and human lung fibroblasts (NHLF). For all 3 fibroblast types, samples cultured under cyclic strain exhibited significant increases in collagen and stiffness relative to static controls, while samples cultured under cyclic strain in combination with vibration did not (Fig. 3.6B). In the final set of studies, we asked if exposure to vibration after previous culture under cyclic strain could promote remodeling of the newly synthesized matrix. When NHDF-seeded sponges conditioned by cyclic strain for one week were cultured an additional week under static conditions, significant
increases in collagen and stiffness compared with one week cyclic strain or static conditions were observed (Fig. 3.6C).

Figure 3.6. Vibration blocks collagen deposition and mechanical stiffening induced by cyclic strain and exogenous TGF-β1. (A) NHDF seeded in 3D, porous polyurethane sponges were subjected to a one-time 20% axial strain and then cultured under static and vibratory conditions (100 Hz, 1.28 ± 0.07 mm amplitude for 6 h/day) with and without exogenous TGF-β1 supplementation (10 ng/ml) for 7 days. (B) 3D, porous polyurethane sponges seeded with NHDF, AoAF, and NHLF were cultured under static conditions, cyclic strain alone (5%, 0.25 Hz), or cyclic strain in combination with vibration (100 Hz, 1.28 ± 0.07 mm amplitude, 3.05 s on/0.95 s off) for 8 h/day for 7 days. (C) NHDF-seeded 3D, porous polyurethane sponges were cultured under cyclic strain (5%, 0.25 Hz) for one week and then subjected to a one-time 15% static strain followed by static or vibratory (100 Hz, 1.28 ± 0.07 mm amplitude for 6 h/day) culture for an additional week. *(P < 0.001) compared with static culture without TGF-β1 (A) or static control (B and C), †(P < 0.001). All graphs, mean ± SD (n = 4).

In contrast, samples subjected to vibration during the second week exhibited significant decreases in collagen and stiffness relative to the one week cyclic strain group, reaching levels not significantly different from the one week static control. In order to
ensure that vibration was not detrimental to the cells, cell number based on DNA content was measured and found not to vary significantly among the groups in any of the three models (Fig. 3.7). We believe these results demonstrate for the first time that high frequency vibration can block critical events in the initiation and progression of fibrotic disease and potentially reverse pathological matrix accumulation in the early stages of disease.

Figure 3.7. Cell number per sponge assessed by DNA content. (A) NHDF seeded in 3D, porous polyurethane sponges were subjected to a one-time 20% axial strain and then cultured under static and vibratory conditions (100 Hz, 1.28 ± 0.07 mm amplitude for 6 h/day) with and without exogenous TGF-β1 supplementation (10 ng/ml) for 7 days. (B) 3D, porous polyurethane sponges seeded with NHDF, AoAF, and NHLF were cultured under static conditions, cyclic strain alone (5%, 0.25 Hz), or cyclic strain in combination with vibration (100 Hz, 1.28 ± 0.07 mm amplitude, 3.05 s on/0.95 s off) for 8 h/day for 7 days. (C) NHDF-seeded 3D, porous polyurethane sponges were cultured under cyclic strain (5%, 0.25 Hz) for one week and then subjected to a one-time 15% static strain followed by either static or vibratory (100 Hz, 1.28 ± 0.07 mm amplitude for 6 h/day) culture for an additional week. All bar graphs, mean ± SD. ns, nonsignificant

3.5. Discussion

Connective tissue throughout the body experiences cyclic tensile loading during routine locomotion at relatively low frequency (≤ 1 Hz) and high magnitude (5-10%
Stromal matrix, composed primarily of type I collagen, provides resistance to these loads. In healthy tissue, fibroblasts balance new matrix synthesis and proteolytic degradation to achieve tensional homeostasis [317]. Mechanobiology plays a central role in maintaining this equilibrium. Numerous studies have shown that exposure to tension, either externally applied or endogenously generated by culture within restrained gels or on relatively stiff surfaces promotes matrix synthesis, while shielding from external loads or exposure to highly compliant substrates accelerates matrix degradation [38,44,222]. One of the central mechanisms underlying this response is tension-induced increases in both the expression and activation of TGF-β1 [335,336]. Particularly in the case of collagen, interference with TGF-β1 activity or signaling has been shown to block tension-induced increases in collagen expression [232]. CTGF expression has also both been shown to be sensitive to mechanical tension, however, it is not always clear if this is direct response or a consequence of strain-induced TGF-β1 expression, for which CTGF is a prominent downstream target [337].

In the present study, we examined the transcriptional response to vibratory stimulation, which, compared to most models of externally applied tension reported in the literature, is characterized by relatively high frequency (100 Hz) and low strain (<1.65% in this study). Although vibration caused significant and sustained increases in mRNA and protein expression of profibrotic cytokines, expression levels of the major fibrillar collagens implicated in fibrosis (Type I, III, and V) were not significantly affected even after 7 day culture. While few previous studies have examined the effect of vibration on cytokine expression, several have examined type I and III collagen expression and also
observed no significant change or down-regulation [324,326–329]. One exception is the study by Wolchok et al. that observed significant increases in TGFB1, CTGF, and collagen type I, as well as increased mechanical stiffness of thin polyurethane foams, however, the experimental design was substantially different since a circular sample geometry was used with complete circumferential fixation and 2D culture for gene expression studies [338]. Although the microarray study did not reveal differential expression of any of the major collagenolytic proteases, we investigated MMP1 expression by qRT-PCR since it had previously been reported to be responsive to vibration and found significant increases at several time points. The potential fibrotic activity of the differential expression observed for TIMP2 and TIMP3 is unclear. While TIMPs have historically been considered profibrotic due to their ability to inhibit proteases that degrade matrix, recent studies have reported increased fibrosis in TIMP-2 and TIMP-3 knockout models, suggesting a more complex role for TIMPs than originally thought [339]. Collectively, these results suggest that vibration exhibits potential antifibrotic activity by inhibiting the induction of collagen gene expression normally activated by profibrotic cytokines and potentially stimulating matrix degradation through induction of MMP1.

Evaluation of TGF-β signaling pathway genes significantly affected by vibration suggested a possible explanation for how profibrotic cytokine activity might be suppressed. Among a wide range of gene targets involved in ligand binding, intracellular signal transduction, transcriptional activation/repression, and negative feedback, an overall pattern of changes in gene expression consistent with inhibition of TGF-β
signaling was observed. First, vibration negatively regulated ligand-receptor interactions by significantly decreasing expression of TGFBR1 and TGFBR2, as well as TGFBR3 that binds TGF-β1 and TGF-β2 and increases their affinity for TGF-β receptor type II [340]. Second, vibration reduced the potential for intracellular signal transduction by decreasing expression of receptor-regulated Smads (R-Smads, SMAD2 and SMAD3) and the Co-Smad, SMAD4. In addition, vibration increased expression of prostate transmembrane protein, androgen induced 1 (PMEPA1), a cytoplasmic protein that can bind and sequester both inactive and active R-Smads, and the inhibitory Smad, SMAD7, which competes with R-Smads for binding to activated receptors [341,342]. Third, vibration increased expression of ubiquitin ligases (SMURF1, SMURF2, and WWP1) and SIK1 that down-regulate TGF-β signaling by targeting R-Smads and TGF-β receptor type I for proteosomal degradation [139,343–345]. Finally, vibration down-regulated expression of the transcriptional co-activator cAMP response element-binding protein 1 (CREBBP), while increasing expression of the transcriptional repressors Smad nuclear interacting protein 1 (SNIP1) and SKIL [346–348]. Overall, these results suggest that vibration down-regulates cellular capacity for TGF-β signal transduction.

TGF-β is considered a key player in the pathogenesis of multiple fibro-proliferative disorders due to its overexpression in diseased tissue and ability to stimulate both matrix synthesis and myofibroblast differentiation [24]. Although a variety of strategies for inhibition of TGF-β activity have shown promise in animal models, success in human clinical trials has been limited [349,350]. In addition, due to its multiple roles in immunomodulation, tumor suppression, and wound healing, prolonged suppression of
TGF-β activity to treat chronic disease may have detrimental side effects [24]. A mechanically-based antifibrotic therapy could offer several substantial benefits relative to existing pharmaceutical approaches, including localized/regional as opposed to systemic activity and ease of dose adjustment and discontinuation that will likely offer improved safety and reduced systemic side effects. As a first step towards establishing proof-of-concept for high frequency vibration as a mechanomodulatory therapy, we showed that vibration can inhibit collagen accumulation and mechanical stiffening in a 3D culture model in response to exogenous TGF-β1 and cyclic strain as models of profibrotic stimuli. While we have primarily focused on dermal fibroblasts since the skin is vulnerable to several fibrotic disorders and the most easily accessible target for mechanotherapy, the demonstration of similar responses in cardiac and lung fibroblasts suggests vibration may have widespread application. Several mechanically-based therapies utilizing high frequency stimulation such as shock wave therapy, low intensity pulsed ultrasound, and low magnitude, high frequency whole body vibration are reaching clinical application [183,266,351]. Currently, these treatments are primarily focused on increasing osteogenesis to promote bone healing and counteract osteoporosis and accelerating angiogenesis to improve graft survival. To our knowledge, this is the first study demonstrating the ability of high frequency mechanical stimulation to counteract increased matrix synthesis in response to profibrotic stimuli.

3.6. Conclusion

In conclusion, these studies show that while vibration increases the expression of profibrotic cytokines, it simultaneously modulates the expression of multiple components
of the TGF-β signaling pathway with a net effect of inhibiting capacity for signal transduction. When applied in combination with profibrotic stimuli, vibration inhibits collagen synthesis and mechanical stiffening. This approach, while prophylactic, may have clinical relevance to pathologies such as radiation-induced fibrosis. The ability of vibration to reduce collagen and stiffness after prior exposure to cyclic strain, suggests potential for intervention in the early stages of disease progression, although it is important to acknowledge that the newly synthesized matrix induced by cyclic stain in our model likely lacks the maturity, organization, and crosslinking of accumulated collagen in clinical fibrosis. Another limitation of the present study is that while we have focused on the fibroblast as the key effector cell in all fibrotic disorders, the complex, multicellular pathology of fibrosis cannot be fully emulated by in vitro models. In order to advance this work, future studies will further test the ability of vibration to reverse the fibrotic phenotype of fibroblasts derived from patients with established disease such as systemic sclerosis and idiopathic pulmonary fibrosis and evaluate its efficacy in animal models of fibrotic disease.
4.1. Abstract

Mechanical stimuli are critical modulators of extracellular matrix (ECM) synthesis and remodeling adhering to the structural and functional needs of the specific tissues. Understanding of changes in ECM molecules by fibroblasts led by vibratory mechanical forces provides how the vocal folds maintain and develop vocal fold-specific matrices in response to vibratory stresses and strains. Fibroblast-seeded elastomeric substrates were housed in vibratory bioreactors and cultured under various vibrational parameters including amplitude, frequency, and duration. Overall, vibratory mechanical stimulation exhibited a significant alteration in the mRNA expressions of vocal fold-related ECM molecules and cytokines regulating ECM metabolism. The gene expression of HA synthase 1 (HAS1), HA synthase 2 (HAS2), hyaluronidase 2 (HYAL2), TIMP3, TGFβ2, CTGF, and endothelin-1 (EDN1) was significantly increased, whereas COL1A1 and COL3A1 were significantly suppressed, suggesting that the expression profiles of genes induced by vibration are similar to physiologically relevant vocal fold ECM composition. Interestingly, our results show that the mRNA expression patterns are significantly responsive to increasing doses of vibrational loadings and altered by different levels of amplitudes or frequencies. These studies demonstrated that vibration can stimulate the tissue-specific mRNA expression to support the vocal fold
microenvironment and will provide specific guidance for the development of a clinical mechanotherapy approaches in the context of vocal fold wound healing and regeneration.

4.2. Introduction

Cells constantly experience dynamic mechanical loads, including compression, shear stress, tensile strain, hydrostatic pressure, and interstitial fluid flow. Through various mechanotransduction processes, these stimuli activate intracellular signaling pathways that influence many aspects of cell behavior including proliferation, differentiation, extracellular matrix (ECM) deposition, soluble factor production, and tissue development [352]. One of the seminal discoveries in mechanobiology has been the ability of physiologically-relevant mechanical stimulation to activate tissue-specific matrix gene expression. For example, dynamic compression promotes the synthesis of sulfated glycosaminoglycans (S-GAG) and improves the growth and mechanical properties of cartilaginous tissues [323]. Cyclic strain stimulates collagen expression and synthesis in cardiac, mesangial, periodontal, pulmonary, tendon, and ligament fibroblasts and elastin synthesis in smooth muscle cells [231,321,353]. These forms of relatively high amplitude, low frequency stimulation have been widely applied in bioreactor systems to increase the mechanical properties in engineered tissues. Alternatively, relatively low amplitude, high frequency mechanical stimulation in the form of whole body vibration (WBV) [354,355], low-intensity pulsed ultrasound (LIPUS) [356,357], and extracorporeal shock wave [258,259,358] have been used clinically to improve both bone and soft tissue healing. However the underlying mechanisms responsible for these effects are not well understood.
The vocal folds are unique laryngeal connective tissues that routinely experiences high frequency vibration during voice production [324]. Several characteristics of the superficial layer of the lamina propria (LPs) within the vocal folds and its relation to the vibratory microenvironment have potential relevance to wound healing. First, the ECM of the native LPs contains relatively high levels of GAGs/proteoglycans (hyaluronan, versican, decorin, and fibromodulin), many with anti-scarring activity, and relatively low levels of fibrous matrix proteins including collagen and elastin. Histological identification of macrophages and myofibroblasts in the LPs of healthy subjects suggests that normal phonation causes LPs microtrauma that is repaired without substantial alterations in matrix composition. Several recent studies have shown that vibration regulates fibroblast expression of many genes encoding matrix constituents relevant to LPs physiology, including upregulation of hyaluronan synthase 1 (HAS1) and HAS2 in human laryngeal fibroblasts [324,327] and increased expression of HAS3 in human neonatal foreskin fibroblasts [329], but reduced expression of collagen type I [325,329]. In addition, we have recently shown that high frequency vibration is able to inhibit the TGF-β signaling pathway and block cyclic strain- and TGF-β-mediated collagen accumulation and mechanical stiffening. Therefore, further understanding of the mechanisms of vibratory mechanotransduction may offer the opportunity to development mechanotherapies capable of modulating wound healing and preventing fibrosis.

Despite the clinical success of mechanotherapies, a commonly cited weakness is the limited information available with respect to optimal parameters and doses of treatment [359,360]. Several studies suggest that a mechanical stimulus is composed of
multiple parameters that induce different levels of molecular and cellular responses [231, 358, 361]. Specifically, the effect of cyclic tension variables such as strain, pulse shape, and pulse frequency has been reported among different cells types and experimental designs [231, 361]. Tranquillo et al. have investigated the effect of different regimes of cyclic strain on collagen expression of smooth muscle cells (SMCs) and found that each of the parameters affected the levels of elastin and collagen production and mechanical strengthening of collagen constructs compared to statically cultured controls [361]. Joshi and Webb also examined dose-dependent responses of cell/scaffold stiffness in response to variable cyclic strain amplitude, rate, frequency, and duration, and observed significant differences in mechanical properties [231]. In addition, shock waves are defined by multiple parameters including the number of impulses, the frequency of impulses, and the density of energy fluxes. Many clinical studies have been conducted to better characterize and optimize the shock wave therapies in numerous groups of wounds [358]. Thus, an improved understanding of the relationship between the characteristics of applied vibratory loading and resulting cellular responses is an important consideration in the development of a vibratory mechanotherapy.

Vibration can be described by three principle characteristics (frequency, amplitude, and duration) [324]. Biomechanically, vocal fold mucosa experiences the vibration at high frequency (100–200 Hz) and deforms approximately 1 mm laterally during each vibration cycle. Vocalization times can vary between ~1-2 hrs per day for normal voice user, while heavy voice users like classroom teachers and opera singers can speak for ~6-7 h per day [325, 362]. In addition, the normal phonatory cycle consists of
alternating periods of vibration and rest and vocal tissues normally receive a long rest period each day during sleep. In this study, we characterized the effect of systematically varying these vibratory loading characteristics (frequency, amplitude, and duration) on ECM-related gene expression in human dermal fibroblasts.

4.3. Materials and Methods

4.3.1. Fabrication of elastomeric sponges

Porous polyurethane sponges were fabricated as previously described [363]. Briefly, Tecoflex SG-80A (4.05 gm; Lubrizol Corporation, Wickliffe, OH) was dissolved in dimethylacetamide (39.1 ml; EMD Millipore, Billerica, MA) overnight at 70 °C. Pluronic 10R5 (18.95 ml; BASF, Florham Park, NJ) was slowly added and the polymer solution was then stirred for 4 hours. The temperature was slowly lowered to 56 °C and the phase-separated mixture was pipetted into delrin molds (3 mm deep x 10 mm wide x 100 mm length). The molds were immediately transferred to a cold ethanol bath, cooled for 2 minutes, and then submerged in distilled water overnight to precipitate the Tecoflex sponge and remove the Pluronic. The substrates were removed from molds, stirred gently in distilled water for 3 days with five additional water changes to completely remove any remaining solvents/Pluronic, and then lyophilized for 3 days.

4.3.2 Vibratory bioreactor

The vibratory bioreactor module consisting of a bioreactor platform equipped with an electromagnetic voice coil actuator (BEI Kimco, Vista, CA) and a modified T-75 flask was constructed as previously described [324]. A sinusoidal waveform signal from a frequency generator (4040A, B&K Precision, Yorba Linda, CA) was processed through
a 10W amplifier (CK003, Cana Kit, North Vancouver, Canada), and transmitted to the input terminal of a reed switch connected to the voice coil actuator. The reed switch was connected to a collector terminal (+5 V from a PC) of a NPN transistor, the base terminal of which was connected to the output port from a custom Labview program (National Instruments, Austin, TX) running on a PC. The vertical displacement of the voice coil was translated into horizontal displacement of a plastic tube holding 4 plastic rods that passed through flexible gaskets into the interior of the T75 flask upon which one end of each sample was mounted. The other end of each sample was mounted on a fixed support pin on the other side of the flask. The frequency and magnitude of the sine-wave oscillation were controlled by the function generator. Total duration, active vibration intervals (‘on’ and ‘off’ times), and inactive (rest) time per day were controlled by the custom Labview software program (National Instruments).

4.3.3. Fibroblast seeding in porous tecoflex sponges

Porous polyurethane substrates (25 mm in length × 3 mm in thickness × 5 mm in width) mounted on a polyester frame using a UV curable adhesive (Loctite 3311) were sterilized by immersion in 70% ethanol for 15 min and washing three times with sterile distilled water, then incubated overnight in 20 µg/ml fibronectin (Sigma, St. Louis, MO) in Dulbecco’s phosphate-buffered saline (PBS). For cell seeding, fibronectin-coated polyurethane substrates were suspended on the two delrin support bars and incubated at 37 °C for 30 minutes. Adult normal human dermal fibroblasts (NHDFs, Lonza, Allendale, NJ) at passages fifth and sixth were trypsinized and re-suspended at 1 x 10⁷ cells/ml. Two 20 µl aliquots of cell suspension were seeded on the top surface of each substrate,
incubated 30 min at 37 °C, and then the samples were turned over and seeded on the opposite side in an identical manner. After 30 min, this procedure was repeated once more with a second seeding onto the top of the substrate, leading to a final seeding density of 1.2 x 10^6 cells/substrate. The substrates were placed in a 100 mm Petri dish containing DMEM/F12 media (Mediatech, Herndon, VA) with 10% BGS (Hyclone, Logan, UT) and 1% penicillin-streptomycin (Mediatech) and cultured for 4 days.

Table 4.1. Variable vibration regimes characterized by varying amplitude, frequency, and duration

<table>
<thead>
<tr>
<th>Variables</th>
<th>Voltage (Vrms)</th>
<th>Frequency (Hz)</th>
<th>Amplitude (mm)</th>
<th>Duration (h)</th>
<th>On/Off Cycle (s/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude</td>
<td>1.9</td>
<td>100</td>
<td>0.77 ± 0.02</td>
<td>6</td>
<td>Constant</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>100</td>
<td>1.28 ± 0.07</td>
<td>6</td>
<td>Constant</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
<td>100</td>
<td>1.61 ± 0.03</td>
<td>6</td>
<td>Constant</td>
</tr>
<tr>
<td>Frequency (Varying Amplitude)</td>
<td>1.5</td>
<td>25</td>
<td>1.25 ± 0.04</td>
<td>6</td>
<td>Constant</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>50</td>
<td>1.27 ± 0.05</td>
<td>6</td>
<td>Constant</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>100</td>
<td>1.28 ± 0.07</td>
<td>6</td>
<td>Constant</td>
</tr>
<tr>
<td>Frequency (Constant Amplitude)</td>
<td>3.8</td>
<td>25</td>
<td>1.98 ± 0.03</td>
<td>6</td>
<td>Constant</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>50</td>
<td>1.84 ± 0.01</td>
<td>6</td>
<td>Constant</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>100</td>
<td>1.28 ± 0.07</td>
<td>6</td>
<td>Constant</td>
</tr>
<tr>
<td>Duration</td>
<td>3.8</td>
<td>100</td>
<td>1.28 ± 0.07</td>
<td>2</td>
<td>Constant</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>100</td>
<td>1.28 ± 0.07</td>
<td>4</td>
<td>Constant</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>100</td>
<td>1.28 ± 0.07</td>
<td>6 h + 18 h rest</td>
<td>Constant</td>
</tr>
<tr>
<td>Active/Rest Period</td>
<td>3.8</td>
<td>100</td>
<td>1.28 ± 0.07</td>
<td>6</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>100</td>
<td>1.28 ± 0.07</td>
<td>6</td>
<td>4/2</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>100</td>
<td>1.28 ± 0.07</td>
<td>6</td>
<td>6/0 (Constant)</td>
</tr>
</tbody>
</table>

*6 h vibrotary culture followed by 18 h resting time.

4.3.4. Vibratory culture

After 4 days of static incubation, NHDF-seeded polyurethane substrates were transferred to the vibratory bioreactor. For vibratory culture, the substrates (n = 4 per group) were subjected to a one-time 20% axial strain and cultured under various vibratory regimes, while control samples were subjected to a one-time 20% axial strain followed by static cultured or maintained under static conditions. The specific parameters that were investigated in this study are shown at Table 4.1. In general, each parameter
was individually varied while the others were held constant using our standard vibration regime (3.8 Vrms, 100 Hz, 6 h) as a baseline. The displacements of porous polyurethane substrates at the driving point were measured at the frequency of 25-125 Hz and the voltage of 1.9, 3.8, and 4.6 Vrms using a digital stroboscope (Fig. 4.1).

Figure 4.1. Displacement of porous polyurethane substrates in the vibratory bioreactor. The substrate displacement was measured at the driving point of polyurethane substrates vibrating at variable frequency (25–125 Hz) and variable voltage (1.9–4.6 Vrms) using a digital stroboscope.

4.3.5. mRNA expression analysis

After vibratory culture, the samples (n = 4) were immediately minced in 1 mL TRIzol reagent (Thermo Fisher Scientific, Waltham, MA), homogenized, and centrifuged at 14,825 rcf for 15 min at 4 ºC. After centrifugation, 0.2 mL chloroform (Acro Organics, Morris Plains, NJ) was added to the supernatant previously transferred to a microcentrifuge tube, and the mixture was agitated, incubated for 10 min, and centrifuged at 14,825 rcf for 15 min at 4 ºC. Subsequently, total RNA was isolated from the aqueous
phase using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The quality and quantity of isolated total RNA were determined using a Take3 Micro-volume plate (BioTek Instruments, Winooski, VT) and a microplate reader (BioTek Instruments, Winooski, VT). 1 µg of total RNA from each sample was used to synthesize cDNA using RETROscript kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Real-time RT-PCR was performed with Quantitect SYBR green PCR Kit (Qiagen) using custom-designed sense and anti-sense primers (Table 4.2) in a Rotorgene 5000 thermal cycler (Qiagen). Relative expression levels of target genes were quantified by the $2^{-\Delta\Delta C_t}$ method, using beta-2-microglobulin (B2M) as an internal standard [364] and expressed as relative fold changes compared to the static control group.

Table 4.2. Primer sequences use in Real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>GeneBank no.</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAS1</td>
<td>TGGTCTTCTGCCTGCTCTAC</td>
<td>CAGAGGGACCTAGTTAGCGG</td>
<td>NM_001523.2</td>
<td>137</td>
</tr>
<tr>
<td>HAS2</td>
<td>CATAAAGAAGCTGCACACAG</td>
<td>CACACTTCGTCGCCGCTCTC</td>
<td>NM_005328.2</td>
<td>126</td>
</tr>
<tr>
<td>HYAL2</td>
<td>CACTTCCCGCTTGTCTACC</td>
<td>AAGACGATGACCGGAGTGC</td>
<td>NM_003773.4</td>
<td>138</td>
</tr>
<tr>
<td>COL1A1</td>
<td>TGCTGGTTCGCTCTTGACTCT</td>
<td>TCCAGAGGGACCTTTGCTG</td>
<td>NM_000088.3</td>
<td>142</td>
</tr>
<tr>
<td>COL3A1</td>
<td>GGATCGCCGAGCTTGGAATG</td>
<td>GTGCAACCATACCTCCAGAAG</td>
<td>NM_000090.3</td>
<td>117</td>
</tr>
<tr>
<td>MMP1</td>
<td>GTGGGAAACCCTGAGTGA</td>
<td>CTTGGGAAATCTCTGGCTGA</td>
<td>NM_001145938.1</td>
<td>141</td>
</tr>
<tr>
<td>TIMP3</td>
<td>TCCCTGACTACCTGGCCCTG</td>
<td>CAGGCCGTAGTGTGGAGCTG</td>
<td>NM_000362.4</td>
<td>107</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>AAACCGACATCTCGTGCTG</td>
<td>GCAGCAAGGAGAGCAGATG</td>
<td>NM_001135599.3</td>
<td>117</td>
</tr>
<tr>
<td>CTGF</td>
<td>TGCTTCTGCACTAACTGCTAAG</td>
<td>TCACTTGGACACAGCTGCC</td>
<td>NM_001901.2</td>
<td>141</td>
</tr>
<tr>
<td>EDN1</td>
<td>CAGAAACAGTCTTAGCCGCTG</td>
<td>TATCCATCGGAGCAGAAG</td>
<td>NM_001168319.1</td>
<td>123</td>
</tr>
<tr>
<td>B2M</td>
<td>TGTTCCGCCGCTACTCTTCTC</td>
<td>CGATGGATGAACCCAGAC</td>
<td>NM_000408.2</td>
<td>137</td>
</tr>
</tbody>
</table>

*All primer sequences were designed based on gene sequence obtained from the respective GenBank numbers using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Abbreviation: HAS, hyaluronan synthase; HYAL2, hyaluronidase 2; COL, collagen; MMP1, matrix metallopeptidase 1; TIMP3, tissue inhibitor of metalloproteinases 3; TGFβ2, transforming growth factor-β2; CTGF, connective tissue growth factor; EDN1, endothelin 1; B2M, beta-2-microglobulin.
4.3.6. Statistical analysis

For the studies to determine the effects of vibratory stimulation with variable amplitude, frequency, and ‘on/off’ time cycle on fibroblast ECM expression, statistical multiple comparisons were performed using a modified Tukey-Kramer method for unequal-sized groups, particularly between 1X Strain and other experimental groups. For the studies to identify whether ECM expression in response to vibratory stimulation is dose-dependent and loading-specific response, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for statistical analysis with multiple comparisons. $P$ values less than 0.05 were considered significant. All quantitative data are expressed as mean ± standard deviation.

4.4. Results

4.4.1. Effect of variable amplitude on fibroblast ECM expression

Fibroblast-seeded porous sponges were cultured under static, one-time 20% strain, or one-time 20% strain and vibratory loading with different amplitudes generated by varying the voltage applied to the voice coil actuator. mRNA expression levels of HAS1, HAS2, HYAL2, TIMP3, TGFB2, CTGF, and EDN1 were significantly up-regulated in response to vibration at all amplitudes and MMP1 at the highest amplitude only compared to the 1X Strain control (Fig. 4.2). COL1A1 was significantly up-regulated at the lowest amplitude only, while COL3A1 was significantly decreased at the two higher amplitudes. When expression levels were compared between variable amplitude groups, the gene expression of HAS1, HAS2, MMP1, TIMP3, TGFB2, CTGF,
and EDN1 were significantly increased at the highest vibratory amplitude, while COL1A1 and COL3A1 were significantly reduced at the highest vibratory amplitude.

![Figure 4.2](image.png)

Figure 4.2. Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either 1X Strain or 1X Strain + Vibration with variable voltage (1.9–4.6 Vrms) and fixed frequency (100 Hz) relative to static control. n = 3 samples per group. *(P < 0.05) compared with 1X Strain group, †*(P < 0.05) statistical comparisons among variable vibration groups. All bar graphs, mean ± SD.

4.4.2. Effect of variable frequency on fibroblast ECM expression

Since vibratory frequency and amplitude are inter-dependent, in order to account for this, two variable frequency studies were performed: one in which the voltage was adjusted to compensate and maintain constant amplitude at varying frequencies and another one in which amplitude was allowed to change alongside frequency. When the substrates were cultured under variable frequency vibration with constant amplitude, a
significant up-regulation of HAS1, HYAL2, TIMP3, CTGF and EDN1 were observed in response to vibration at all frequencies relative to 1X Strain control (Fig. 4.3).

Figure 4.3. Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either 1X Strain or 1X Strain + Vibration with fixed amplitude (approximately 1.28 ± 0.07 mm in amplitude) defined by variable frequency (25–100 Hz) and variable voltage (1.5–3.8 Vrms) relative to static control. n = 3 samples per group. *(P < 0.05) compared with 1X Strain group, †(P < 0.05) statistical comparisons among variable vibration groups. All bar graphs, mean ± SD.

mRNA expression levels of HAS2 were significantly increased at the two highest frequencies and TGFB2 at the highest frequency only compared to the 1X Strain control. COL1A1 and COL3A1 were significantly decreased at all frequencies compared with 1X Strain control. When expression levels were compared between variable frequency groups, gene expression of HAS2, TIMP3, TGFB2, CTGF, and EDN1 were significantly
increased at the highest vibratory frequency, while HAS1 was significantly elevated at the lowest vibratory frequency. No significant differences in mRNA expression levels of COL1A1, COL3A1, and MMP1 were not observed in response to variable vibratory frequency groups.

Figure 4.4. Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either 1X Strain or 1X Strain + Vibration with variable amplitude (0.64−1.98 mm in amplitude) defined by variable frequency (25−100 Hz) and fixed voltage (3.8 Vrms) relative to static control. n = 3 samples per group. *(P < 0.05) compared with 1X Strain group, †(P < 0.05) statistical comparisons among variable vibration groups. All bar graphs, mean ± SD.

In the second variable frequency study in which amplitude was allowed to freely change, mRNA expression levels of HAS1, HAS2, HYAL2, TIMP3, TGFB2, CTGF, and EDN1 were significantly increased in response to vibration at all frequencies and MMP1 at 50 Hz frequency only compared to the 1X Strain control (Fig. 4.4). In addition,
COL1A1 was significantly elevated at the lowest frequency and reduced at the highest frequency compared to 1X Strain control. COL3A1 was also significantly decreased at all frequencies. When expression levels were compared between variable frequency groups, significant increases in mRNA expression levels of TGFB2 and EDN1 were observed at the highest frequency, while HAS2, COL1A1, and CTGF were significantly higher at the lowest frequency.

4.4.3. Effect of variable total duration on fibroblast ECM expression

To investigate the effect of vibration exposure time on gene expression, fibroblast-seeded sponges were exposed to vibration for 2, 4, and 6 h. Moreover, since we applied 1X tension at the time of substrate loading, additional groups, 1X Strain group (2, 4, and 6 h) were included in order to clearly observe the effect of vibratory stimulation alone. We found that there was no significant differential gene expression induced by one-time 15% tensioning at all time point compared to static control, except for MMP1 at 2 h, TGFB2 at 6 h, and CTGF and EDN1 at the 2 and 4 h time point (Fig. 4.5). Interestingly, however, mRNA levels of all targets induced by 1X Strain were returned to the levels that were not significantly different to those of statically cultured group except for TGFB2. Relative to 1X Strain controls, vibration leads to a significant increase in the mRNA expression levels of HAS1, HYAL2, CTGF and EDN1 at all time points and HAS2, MMP1, TIMP3, and TGFB2 at the two highest time points. However, COL1A1 at 2 and 6 h and COL3A1 at 4 and 6 h were significantly decreased. When the mRNA levels were compared among varying time duration groups, HAS1, CTGF, and EDN1 notably exhibited the highest expression levels at the 2 h time point. Other targets including
HAS2, HYAL2, MMP1, TIMP3, and TGFB2 showed significantly higher expression levels at the 4 and 6 h time points relative to the 2 h time point.

Figure 4.5. Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either Static, 1X Strain, or 1X Strain + Vibration with variable duration (2, 4, and 6 hours) at fixed frequency (100 Hz) and fixed voltage (3.8 Vrms) relative to static control. n = 3 samples per group. *(P < 0.05) compared with 1X control at each time point, †(P < 0.05) ) statistical comparisons among variable vibration groups. All bar graphs, mean ± SD.

4.4.4. Effect of variable active/rest (ON/OFF) periods on fibroblast ECM expression

The normal phonatory cycle consists of repetitive vibration and rest periods. To evaluate the effect of varying active/rest periods of vibratory patterns on ECM-related gene expression, the substrates were cultured under three different time cycles
(vibrated/non-vibrated, ON/OFF, sec/sec): 2/4, 4/2, and 6/0 (constant) for total 6 h. The results demonstrated that the mRNA expression levels induced by vibratory stimulation is dose-dependent. The vibratory patterns with different time periods exhibited a significant increase in HAS1 and CTGF in response to vibration at all cycle patterns, while mRNA expression levels of HAS2, HYAL2, TIMP3, TGFB2, and EDN1 were significantly up-regulated at the two higher dosing cycles and MMP1 at the two lower dosing cycles (Fig. 4.6).

Figure 4.6. Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to either 1X Strain or 1X Strain + Vibration with variable time cycle (e.g. 2 second on/4 second off in total 6 sec (2s/4s), 4s/2s, and 6s/0s) at fixed frequency (100 Hz) and fixed voltage (3.8 Vrms) relative to static control. n = 3 samples per group. *(P < 0.05) compared with 1X Strain group, †(P < 0.05) statistical comparisons among variable vibration groups. All bar graphs, mean ± SD.
However, a significant down-regulation in COL1A1 at the highest and lowest dosing cycles and COL3A1 at the two lower dosing cycles were observed in response to vibration relative to 1X Strain control. When expression levels were compared between variable vibratory time cycle groups, mRNA expression levels of HAS2, HYAL2, TIMP3, TGFB2, CTGF, and EDN1 were significantly increased with increasing total vibration, while MMP1 was significantly reduced with increased total vibration. Expression of COL1A1 and COL3A1 were either not significantly different or significantly lower than 1X strain control under all vibratory conditions.

4.4.5. Changes in gene expression after discontinuation of vibration and re-exposure

Lastly, we tested whether the changes in gene expression elicited by vibration were a transient or long-lasting response. In addition to 1X strain control and vibration for 6 h, two additional groups were added: (a) 6 h vibration followed by 18 h rest period and (b) 6 h vibratory culture for 3 days. Significant changes in gene expression levels were observed when mRNA expression levels were compared between 6 h vibrated and 6 h vibration followed by further 18 h static culture (Fig 4.7). HAS1, HAS2, HYAL2, MMP1, TIMP3, TGFB2, CTGF, and EDN1 were significantly downregulated at, by more than 7-folds, 2-folds, 5-folds, 0.5-folds, 2-folds, 9-folds, 5-folds, and 9-folds, compared with 6 h vibration groups, respectively. By contrast, COL1A and COL3A1 were significantly up-regulated after 6 h vibration followed by 18 h static culture, by approximately 2-folds, compared with 6 h vibration groups. In addition, when the substrates seeded with fibroblasts were vibrated for 6 h per day up to 3 days, we found that the mRNA expression levels of HAS1, HAS2, TIMP3, and CTGF were significantly
increased at day 3 compared with one day 6 h vibration group, while the levels of HYAL2, MMP1, TGFβ2, and EDN1 mRNAs remained up-regulated at day 3, although not significantly different compared to one day 6 h vibration group. Moreover, the levels of COL1A1 and COL3A1 remained significantly downregulated under vibratory culture over 3 days.

Figure 4.7. Transient response of vibratory stimulation on the gene expression of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines, but responsive to vibration (6 h/day) for 3 days. Relative mRNA expression levels of various matrix proteins, matrix remodeling enzymes, and profibrotic cytokines in response to Vibration (6 h), Vibration (6 h) followed by resting period (18 h), or Vibration (6 h/day) for 3 days at fixed frequency (100 Hz) and fixed voltage (3.8 Vrms) relative to static control. *$n = 3$ samples per group. *($P < 0.05$) compared with static control, †($P < 0.05$) statistical comparisons between 6 h vibration and 6 h vibration + 18 h resting or between 6 h vibration at one day or at 3 days. All bar graphs, mean ± SD.
4.5. Discussion

Mechanical cues are critical in maintaining and developing the microenvironment of living cells and tissues, and the cellular and molecular responses to mechanical stimuli has been widely investigated for induction of tissue-specific matrix synthesis. In this study, using an in vitro vibratory bioreactor, we cultured human dermal fibroblasts under variable vibratory patterns defined by three main characteristics (amplitude, frequency, and duration) and then analyzed the molecular response to vibratory stimulation through the mRNA analysis of ECM-related and cytokine genes. The objective was to gain fundamental insight into the sensitivity of changes in gene expression to changes in vibratory stimulus and determine if it was possible to identify a particular component of the vibratory pattern that had the strongest influence on gene expression.

With regard to tissue-specific matrix production in response to vibratory stimulation, several studies have focused on differential expression of vocal fold ECM-related genes by human laryngeal and dermal fibroblasts in response to vibratory mechanical stimuli using different bioreactor modules [324,329,338]. Titze et al. investigated the effects of physiologically relevant vibratory stimulation on gene expression by human laryngeal fibroblasts cultured in porous Tecoflex sponges. They showed that vibratory stimulation at 100 Hz for 6 h resulted in a significant increase in fibronectin, MMP-1, and HA synthase 2 (HAS2) compared with those of statically cultured fibroblasts [365]. Farran et al. cultured human neonatal foreskin fibroblasts (NFFs)-seeded silicon membrane under vibratory stimulation at the range of 60–300 Hz with the varying displacement (1–30 µm) for 1 h followed by a 6 h rest. They found that
the gene expression of collagen type I was not significantly elevated at high frequency (≥ 100 Hz), while vibration at below 60 Hz led to a significant increase in collagen type I expression compared with static controls. However, the gene expression of HAS3 and HYAL1 at increased displacement was significantly increased relative to those at lower range of displacements (≤ 5 um). In addition, vibratory stimulation at the frequency of 110 Hz and displacement of 30 mm led to 0.8-folds and 0.9-folds decreases in the mRNA levels of collagen type I and MMP1 compared to the static controls, respectively. Regarding collagen expression, they also reported that lower frequency with decreased amplitude induced elevated collagen type I expression. Moreover, NFF cultured under vibration at 110 Hz with a displacement of 30 mm for 1 h resulted in 20% reduction in collagen type I expression compared to the static culture [329]. Kutty and Webb also subjected human dermal fibroblasts to a vibration (100 Hz in frequency and 5.3 Vrms in voltage) in a 2s/2s (ON/OFF) regimen for 4 h per day over 7 days. They also found that HAS2 and MMP1 expression were significantly increased, while did not lead to significant changes in collagen expression [325].

In a similar manner, our results also demonstrated that vibratory stimulation induced the mRNA expression of vocal fold-specific ECM molecules and cytokines regulating ECM metabolism including HAS1, HAS2, HYAL2, TIMP3, TGFB2, CTGF, and EDN1, whereas COL1A1 and COL3A1 were suppressed. Interestingly, the gene expression of HAS1, HAS2, TIMP3, TGFB2, CTGF, and EDN1 was extremely sensitive to vibratory stimulation, however, the profiles of HYAL2, COL1, COL3A1, and MMP1 were less responsive to vibratory stimulation. Our results also suggest that the molecules
that were responsible for regulating matrix degradation and remodeling can also be modulated by vibration stimulations. Interestingly, MMP1 and TIMP3 look more sensitive to frequency and amplitude changes. In addition, it is suggested that vibratory forces should be applied with a precisely timed manner. Because, a significant change in mRNA expression induced by 6 h of vibratory stimulation cannot be observed after 18 h of resting period after vibratory culture.

As a therapeutic application of vibratory stimulation, this study investigated for the first time the effects of variable vibratory regimes on ECM gene expression and provided a basis how the vibratory stimulation could be formed and shaped to be effective for vocal fold tissue regeneration. Although there are experimental limitations that our vibratory module cannot fully produce more broad range of frequency that correspond to physiological levels (100-300 Hz), our results indicates that high frequency vibration can positively modulate the gene expression of vocal fold ECM against the molecular response of vocal fold injury through increasing HASs and decreasing collagens. Ultimately, the optimal vibratory amplitude and dosing will need to be tested in an animal model.

4.6. Conclusions

Variable vibratory loading regimes induced different levels of ECM-related gene expression in human dermal fibroblasts. HAS1, HAS2, HYAL2, TGFB2, CTGF, and EDN1 exhibited strong sensitivity to changes in vibratory amplitude and duration, while COL1A1 and COL3A1 did not seem to be responsive to vibratory stimulation. The altered mRNA expressions by vibratory stimulation demonstrated by this study are
consistent with ECM composition that is physiologically relevant to vocal fold mucosa: increased HA-related molecules and decreased collagens, despite unexpected induction of cytokines. To be more clinically effective vibratory mechanotherapy against wound healing, we acknowledge that more specific parameters finely-tuned by amplitude, frequency, and duration need to be carefully considered, however, we believe that this study may provide a basic guideline for a potential vibratory therapy in the context of wound healing. The future studies will further test the ability of variable vibration regimes to optimize the induction of tissue-specific ECM production.
CHAPTER FIVE

ATTENUATION OF THE FIBROTIC PHENOTYPE OF SCLERODERMA DERMAL FIBROBLASTS BY VIBRATORY STIMULATION: POTENT ANTIFIBROTIC MECHANOTHERAPY

5.1. Abstract

Fibrosis can lead to permanent functional loss in every major organ, accounting for 45% of deaths in the United States. Recent studies suggest that mechanical forces may be an important therapeutic target that is able to modulate ECM synthesis and enhance wound healing. Previous studies have shown that vibration significantly down-regulated TGF-β signaling and prevented collagen synthesis and accumulation. Moreover, vibration blocked induction of collagen production and mechanical stiffening of polyurethane substrates by profibrotic stimuli for a week. In this study, scleroderma fibroblasts seeded in porous polyurethane sponges were subjected to either further static, cyclic strain (0.25 Hz, 5% strain amplitude) or cyclic strain in combination with vibration under tension (100 Hz frequency, 3.8 Vrms, 1.28 ± 0.07 mm amplitude). qRT-PCR results show that vibration decreased pathological markers (TGFBR2, IL1A, IL4, FBN1, IL1A, IL4, LOX1, TIMP1, COL1A1, and COL3A1 that are over-expressed by scleroderma fibroblasts), and increased antifibrotic regulators (SMAD7 and FLI1) compared with the cyclic strain alone group. In addition, vibration significantly reduced total collagen content and substrate stiffness induced by cyclic strain condition. These studies suggest that vibration may be applicable as a novel mechanotherapy approach for treatment of fibrotic diseases in multiple tissues and organs.
5.2. Introduction

Systemic sclerosis (SSc) is a systemic, connective-tissue disease that eventually leads to pathologic fibrosis in the skin and numerous internal organs. Fibrosis in dermal tissue manifests as the excessive, progressive deposition of collagens and other extracellular matrix (ECM) components due to the aberrant expression of transforming growth factor (TGF)-β accompanied by a severe fibroproliferative vasculopathy and immune system abnormality [119,120,366,367]. Fibroblasts derived from clinically affected skin areas of patients with scleroderma exhibit activated phenotypic markers with aberrant expression of cytokines and growth factors and massive production of extracellular matrix components including Interleukin 1 (IL1) [368], IL4 [369], IL6 [370], connective tissue growth factor (CTGF) [371], endothelin 1 (EDN1) [372], Cartilage Oligomeric Matrix Protein (COMP) [373], tissue inhibitors of metalloproteinases (TIMPs) [374], Fibrillin 1 (FBN1) [375], lysyl oxidase (LOX) [376], and collagens (COL) [374]. Although a variety of strategies against fibrotic disorders have targeted the increased expression of profibrotic cytokines, specifically TGF-β and its downstream molecules which are known to promote collagen synthesis and myofibroblast activation [377–380], success in clinical treatments has been restricted [381].

In addition to soluble signaling factors, mechanical forces have been shown to be able to modulate cytokine and ECM synthesis and secretion in the context of wound healing [37,266,354,355,382,383]. For example, shockwaves are known to promote wound healing through suppressed expression of the pro-inflammatory cytokines such as
IL-1, IL-6, and TNF-α [258]. Several clinical studies also demonstrated that vibration (30–50 Hz) leads to enhanced wound healing by elevated expression of angiogenic factors (VEGF and IGF1) and transition to myofibroblasts with less inflammatory phenotype [384–387]. An increased wound healing was also reported when vibration generated by Vibro-Pulse therapy (Vibrant Medical, Sheffield) applied locally to the wound site [388]. Previously, we have shown that vibratory stimulation may offer a potential mechanotherapy for fibrotic diseases. Human dermal fibroblast-seeded polyurethane substrates subjected to cyclic strain (0.25 Hz) in combination with vibration (100 Hz, 1.28 ± 0.07 mm amplitude) exhibited reduced collagen accumulation and mechanical stiffening of the substrates compared with those cultured under cyclic strain (0.25 Hz) alone. Vibration also significantly inhibited collagen deposition and substrate stiffening induced by exogenous addition of TGF-β. We noted that vibration down-regulated TGF-β signaling via decreased expression of signal transduction molecules (TGFBR2, TGFBR3, and SMAD3) and increased expression of molecules involved in signal inhibition (SMAD7, SMURF1, SMURF2, SIK1, and SKIL).

The purpose of this study was to investigate the antifibrotic effects of vibratory stimulation on scleroderma (SSc) fibroblasts. Here we show that vibratory stimulation (1) induces downregulation of TGF-β signaling in SSc fibroblasts consistent with our previous study in normal fibroblasts, (2) attenuates the expression of scleroderma phenotypic targets, and (3) reduces the increases in collagen accumulation and substrate stiffening by SSc fibroblasts-seeded in polyurethane sponges subjected to cyclic strain.
5.3. Materials and Methods

5.3.1. Polyurethane sponge fabrication

Porous polyurethane sponges were fabricated as previously described [363]. Briefly, Tecoflex SG-80A (4.05 gm; Lubrizol Corporation, Wickliffe, OH) was dissolved in dimethylacetamide (39.1 ml; EMD Millipore, Billerica, MA) overnight at 70 °C. Pluronic 10R5 (18.95 ml; BASF, Florham Park, NJ) was slowly added to Tecoflex solution and the polymer solution was then stirred for additional 4 hours. The temperature was slowly lowered to 56 °C and the cloudy mixture was pipetted into delrin molds (100 mm in length × 10 mm in width × 3 mm in depth). The molds were immediately transferred to a cold ethanol bath, cooled for 2 minutes, and then soaked in distilled water overnight to precipitate the Tecoflex sponge and remove the Pluronic. After 24 h, the polyurethane sponges were removed from molds, washed with distilled water for 2 days to completely remove any remaining solvents/Pluronic, and then lyophilized.

5.3.2. Scleroderma dermal fibroblast culture

Porous polyurethane substrates (25 mm in length × 5 mm in width × 2 mm in height) mounted on a polyester frame using a UV curable adhesive (Loctite 3311) were sterilized by 70% ethanol for 15 min and washed three times with sterile distilled water, then incubated overnight in 20 µg/ml fibronectin (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) at 4 °C with constant mild stirring. For cell seeding, fibronectin-coated polyurethane sponges were suspended on two delrin support bars and incubated at 37 °C for 30 minutes. Human dermal fibroblasts from clinically affected (“SSc”) and clinically unaffected areas (“Normal”) of patients with scleroderma (SSc) were kindly
donated by Dr. Carol A. Feghali-Bostwick (Medical University of South Carolina). Both normal and SSc dermal fibroblasts were cultured and expanded in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin-streptomycin (Mediatech). Both fibroblasts at passages between fourth and sixth were trypsinized and re-suspended at 1 x 10^7 cells/ml. Two 20 µl aliquots of cell suspension were seeded on the top surface of each substrate, incubated 20 min at 37 °C, and then re-seeded on the opposite side in an identical manner. After another 20 min, this procedure was repeated again for the top of the substrate, leading to a final seeding density of 1.2 x 10^6 cells per substrate. The substrates were placed in a T75 flask containing DMEM/F12 media (Mediatech, Herndon, VA) with 10% BGS (Hyclone, Logan, UT) and 1% penicillin-streptomycin (Mediatech) and cultured for 4 days.

5.3.3. Vibratory culture

The vibratory bioreactor module consisted of a bioreactor platform equipped with an electromagnetic voice coil actuator (BEI Kimco, Vista, CA) and a modified T-75 flask as previously described [324]. A sinusoidal waveform signal coming from a frequency generator (4040A, B&K Precision, Yorba Linda, CA) was processed through a 10W amplifier (CK003, Cana Kit, North Vancouver, Canada), and then transmitted to the input terminal of a reed switch connected to the voice coil actuator. The reed switch was connected to a collector terminal (+5 V from a PC) of a NPN transistor and the base terminal of which was connected to the output port from a custom LabView program (National Instruments, Austin, TX) running on a PC. The vertical movement produced by
voice coil actuator was transformed into horizontal displacement that allows to vibrate 4 polyurethane sponges at the same time. The frequency and magnitude of the sine-wave oscillation were controlled by the function generator and total duration controlled by the custom LabView software program (National Instruments). For vibratory culture, fibroblast-seeded polyurethane sponges were transferred to the vibratory bioreactor after 4 days of static incubation. The samples ($n = 4$ per group) were subjected to a one-time 20% axial strain and cultured under vibration (100 Hz, $1.28 \pm 0.07$ mm amplitude for 6 h/day), while control samples were maintained under static conditions.

5.3.4. *In vitro* fibrotic model

In order to model the fibrotic phenotype by stimulating collagen synthesis and mechanical stiffening, scleroderma fibroblast-seeded in porous polyurethane sponges ($1.2 \times 10^6$ cells per sponge) were subjected to profibrotic conditions (cyclic strain) either with or without simultaneous application of vibratory stimulation. Briefly, once the samples were transferred into the vibratory bioreactor, samples were exposed to a one-time, 15% static strain (3 mm strain amplitude) for initial tensioning, followed by 5% axial cyclic strain (1 mm strain amplitude, 0.25 Hz) alone or in combination with vibration under tension (100 Hz, $1.28 \pm 0.07$ mm amplitude, 3.05 s on/0.95 s off) for 8 h/day, as described in section 3.3.9. All samples including controls were cultured in DMEM/F12 media (Mediatech) supplemented with 10% BGS (Hyclone), 1% penicillin-streptomycin (Mediatech), and particularly with 1 mM L-ascorbic acid 2-phosphate (Sigma) to support functional collagen deposition [8].
Table 5.1. Primer sequences used in Real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′−3′)</th>
<th>Reverse Primer (5′−3′)</th>
<th>GeneBank no.</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1</td>
<td>TCCAGCGAGAAGAATCCTGG</td>
<td>TCCAGCGCCAAATGTAGGG</td>
<td>NM_000660.5</td>
<td>137</td>
</tr>
<tr>
<td>CTGF</td>
<td>TGATCTCCGACACATCAAG</td>
<td>TCACCTGCCAAGAGCTGTC</td>
<td>NM_001901.2</td>
<td>141</td>
</tr>
<tr>
<td>EDN1</td>
<td>CAGAAAGACCTTAAAGGCGCTG</td>
<td>TATCCATCGAGGAGACAG</td>
<td>NM_001168319.1</td>
<td>123</td>
</tr>
<tr>
<td>TGFB1</td>
<td>TCAGGATGTACCACCTTGGT</td>
<td>AGCACTCTGCTGACTTGG</td>
<td>NM_001306210.1</td>
<td>148</td>
</tr>
<tr>
<td>TGFB2</td>
<td>GTGCGATGTTGAAATGAGG</td>
<td>TGTTGAAATCTGACTGCA</td>
<td>NM_001024847.2</td>
<td>132</td>
</tr>
<tr>
<td>TGFB3</td>
<td>TCGGACGCAAGACATGATGG</td>
<td>AGCGGCTGAAACTTGATCA</td>
<td>NM_00195683.1</td>
<td>131</td>
</tr>
<tr>
<td>SMAD3</td>
<td>AGTCTGTGGTCAATACCCAT</td>
<td>TGGAATGGCTGACTCTG</td>
<td>NM_00145103.1</td>
<td>123</td>
</tr>
<tr>
<td>SMAD7</td>
<td>TCGGAAATGTGTCGGTGG</td>
<td>AGACTGCGCTGATGTGAG</td>
<td>NM_001190821.1</td>
<td>129</td>
</tr>
<tr>
<td>SKI</td>
<td>GTGCTGCAAAGACATCCTGG</td>
<td>AGCTGATCATTGCTTGG</td>
<td>NM_17354.3</td>
<td>108</td>
</tr>
<tr>
<td>SKL</td>
<td>TTGCTCTTCTGAGGGGCCTC</td>
<td>GTTCCCTTCTTAATGCTGG</td>
<td>NM_001248008.1</td>
<td>137</td>
</tr>
<tr>
<td>SMAD3</td>
<td>AGTCTGTGGTCAATACCCAT</td>
<td>TGGAATGGCTGACTCTG</td>
<td>NM_00145103.1</td>
<td>123</td>
</tr>
<tr>
<td>COL1A1</td>
<td>TGCGCTTGGTCACTCTGGACT</td>
<td>TTCCAGAGGACCTTGTTGG</td>
<td>NM_000088.3</td>
<td>142</td>
</tr>
<tr>
<td>COL3A1</td>
<td>GGATCTGAGCCAGGAAATGT</td>
<td>GTGCAACACTCTTCCAGA</td>
<td>NM_00090.3</td>
<td>117</td>
</tr>
<tr>
<td>IL1A</td>
<td>GCCCCAAGAGTAAGCACAACC</td>
<td>TTATGCTGCTGATGTCC</td>
<td>NM_000575.4</td>
<td>111</td>
</tr>
<tr>
<td>IL4</td>
<td>TACACGACACTTCTGGCCTCC</td>
<td>CGACTGCCTTCTCAATGTG</td>
<td>NM_000589.3</td>
<td>106</td>
</tr>
<tr>
<td>IL6</td>
<td>ATTCAGAAGAGTAGCCGCCGC</td>
<td>TGCTCTTTTCTGCTCTCA</td>
<td>NM_000600.4</td>
<td>149</td>
</tr>
<tr>
<td>FBN1</td>
<td>CTGCAATGACATGCTGTGCGG</td>
<td>TGTTACCTCCACTCTGCC</td>
<td>NM_000138.4</td>
<td>136</td>
</tr>
<tr>
<td>LOX</td>
<td>GATTCTTCCACCAAGGGCCAC</td>
<td>CTCTGCTGTTGCGATCA</td>
<td>NM_00178102.2</td>
<td>113</td>
</tr>
<tr>
<td>TIMP1</td>
<td>CTCTGCCACACTACACTTGCG</td>
<td>CGAGTGGAAAACGCGGAAAC</td>
<td>NM_000254.2</td>
<td>130</td>
</tr>
<tr>
<td>FLI1</td>
<td>CCTCTACACACAGGAGATGG</td>
<td>ATGTGAGATGGTGCTGG</td>
<td>NM_001167681.2</td>
<td>101</td>
</tr>
<tr>
<td>B2M</td>
<td>TGCTCTCAGGCTACTTCTGGC</td>
<td>CGGTGAGATGAAACGAGAC</td>
<td>NM_000408.2</td>
<td>137</td>
</tr>
</tbody>
</table>

*All primer sequences were designed based on gene sequence obtained from the respective GenBank numbers using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Abbreviation: TGFβ1, transforming growth factor-β1; CTGF, connective tissue growth factor; EDN1, endothelin 1; TGFB1, TGF-β receptor; SKI1, salt-inducible kinase 1; SKL, SKI-like proto-oncogene; COL, collagen; IL, interleukin; FBN1, fibrillin 1; LOX, lysyl oxidase; TIMP1, tissue inhibitor of metalloproteinases 1; FLI1, friend leukemia integration 1 transcription factor; B2M, beta-2-microglobulin.

5.3.5. Quantitative RT-PCR analysis

Following vibratory culture, the samples (n = 4) were immediately minced in 1 mL TRIzol reagent (Thermo Fisher Scientific, Waltham, MA), homogenized, and centrifuged at 14,825 rcf for 15 min at 4 ºC. After centrifugation, 0.2 mL chloroform (Acro Organics, Morris Plains, NJ) was added to the supernatant solution previously isolated, and the mixture was then vigorously agitated, incubated for 3 min, and centrifuged at 14,825 rcf for 15 min at 4 ºC. Subsequently, total RNA was isolated from the aqueous phase using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. The quality and quantity of isolated total RNA were
determined using a microplate reader (BioTek Instruments, Winooski, VT) equipped with a Take3 Micro-volume plate (BioTek Instruments). 1 mg of total RNA from each sample was used to synthesize cDNA using RETROscript kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Real-time RT-PCR was performed with Quantitect SYBR green PCR Kit (Qiagen) using custom-designed sense and anti-sense primers (Table 5.1) in a Rotorgene 5000 thermal cycler (Qiagen). Relative expression levels of target genes were normalized by the $2^{-\Delta\Delta Ct}$ method using beta-2-microglobulin (B2M) as an internal standard [364] and expressed as relative fold changes compared to the controls.

5.3.6. Hydroxyproline assay

Collagen accumulation was measured by the hydroxyproline assay modified from the original method described by Woessner et al. [334]. Briefly, polyurethane sponges following vibratory culture were digested in 1 ml of 12 N HCl, boiled at 98 °C for 3 hours, and cooled down to room temperature. After centrifugation, 20 µl aliquots of samples and standard solutions (4-hydroxyproline) were transferred to a 96 well plate, then dried in a vacuum oven overnight. Samples were dissolved in 100 µl of chloramine T solution [141 mg/ml chloramine-T, 1 ml n-propanol and 8 ml OH-Pro buffer (1.25 g citric acid, 3 g sodium acetate, 0.6 g NaOH, dissolved in 30 ml distilled water containing 0.3 ml glacial acetic acid and 7.5 ml n-propanol, pH 6.0)], mixed with 100 µl p-dimethyl amino benzaldehyde solution (0.75 g p-dimethyl amino benzaldehyde in 3 ml n-propanol and 1.3 ml 70% v/v perchloric acid), incubated for 20 min at room temperature, and then heated to 60 °C for 15 min. The absorbance was measured at 558 nm using a microplate
reader (BioTek Instruments). Total collagen content was calculated based on the estimation that hydroxyproline comprises about 13.5% of the total collagen [389]. Total collagen values were normalized to total DNA values quantified using a PicoGreen dsDNA assay kit (Molecular Probes, Eugene, OR). All chemicals and solvents used in this study were provided by Sigma-Aldrich and of analytical reagent grade.

5.3.7. Tensile testing

After vibratory culture, samples were gently washed with PBS and removed from the plastic frames. The width and thickness of samples were measured using a digital caliper. Samples were mounted using aluminum grips covered with Gator P100 sandpaper (Ali Industries, Inc., Fairborn, OH) to prevent slippage. The separation distance between top and bottom grips was set to 5 mm. Using a 100 N load cell, the samples were strained until failure at 1 mm/s using an MTS Synergie 100 (MTS Systems Corporation, Eden Prairie, MN) and data analyzed using Testworks 4 software (MTS Systems Corporation). Construct elastic modulus was calculated by a peak slope method, generally corresponding to the 30% to 60% strain region as previously described [231].

5.3.8. Statistical Analysis

Data analysis was performed by using GraphPad Prism software (GraphPad, San Diego, CA). One-way analysis of variance (ANOVA) with Tukey's post hoc was used for statistical analysis of multiple comparisons. $P$ values less than 0.05 were considered significant. All quantitative data are expressed as mean ± standard deviation.
5.4. Results

5.4.1. Vibration down-regulates mRNA expression of TGF-β signaling molecules in scleroderma fibroblasts

We first characterized differences in expression of TGF-β-related genes between non-lesional (normal) and lesional (SSc) fibroblasts in 1X Strain controls and then how gene expression changed in response to 1X Strain + Vibration after 7 days in culture. With respect to normal fibroblasts, qRT-PCR showed significantly increased expression of the profibrotic cytokines TGFB1, CTGF, and EDN1 in SSc fibroblasts (Fig 5.1A). In addition, consistent with previous studies, vibratory stimulation produced significant increases in expression of CTGF and EDN1 compared with 1X Strain controls in both normal and scleroderma fibroblasts and TGFB1 in SSc fibroblasts only. Expression levels of TGFBR1, TGFBR2, and SMAD3, but not TGFBR3 were also significantly increased in SSc relative to normal fibroblasts (Fig 5.1B). Exposure to vibration resulted in significant decreases in TGFBR2 in SSc fibroblasts and TGFBR3 and SMAD3 in both normal and SSc fibroblasts relative to 1X strain controls. Among three targets involved in inhibition of TGF-β signaling, SMAD7 expression levels were significantly lower in SSc than normal fibroblasts, while SIK1 and SKIL were not significantly different (Fig. 5.1C). However, exposure to vibration significantly increased expression of all three targets in both cell types.
Figure 5.1. Vibration down-regulates TGF-β signaling on SSc fibroblasts. Relative mRNA expression levels of profibrotic cytokines (A) and downstream targets involved in TGF-β signal transduction (B) and inhibition (C). All data were normalized to static control at 7 days. *($P < 0.05$) compared with normal dermal fibroblast group (Normal), †($P < 0.05$) compared with 1X Strain group in each cell. All bar graphs, mean ± SD.
5.4.2. Vibration prevents collagen gene expression, matrix accumulation, and mechanical stiffening by scleroderma fibroblasts

Dermal fibroblasts isolated from lesional skin of scleroderma patients are known to overexpress collagen type I and III compared with those from clinically nonlesional skin [390,391]. qRT-PCR results showed significantly elevated expression of collagens COL1A1 and COL3A1 in SSc fibroblasts relative to normal fibroblasts (Fig. 5.2A). In addition, expression levels of COL1A1 in both SSc and normal fibroblasts and COL3A1
in SSc fibroblasts were significantly reduced by exposure to vibratory stimulation relative to 1X Strain controls. Moreover, total collagen accumulation and mechanical stiffness were significantly higher for SSc fibroblasts than normal fibroblasts under static or 1X strain conditions (Fig. 5.2B). Collagen content and elastic modulus were both significantly reduced in SSc-seeded samples exposed to vibration relative to static and 1X strain conditions and not significantly different from levels observed in normal fibroblasts. Cell number based on DNA content also showed no significant differences among the groups, confirming that vibration did not adversely affect cell viability.

5.4.3. Vibration attenuates the expression of scleroderma phenotypic targets

To investigate the role of vibratory stimulation in modulating fibrotic gene expression, the gene expression of scleroderma phenotypic targets were validated by qRT-PCR. In 1X strain controls, mRNA levels of pro-inflammatory cytokines (Interleukin 1 (IL1A), IL4, and IL6), lysyl oxidase (LOX), and tissue inhibitor of matrix metalloproteinase 1 (TIMP1) were significantly elevated in SSc fibroblasts relative to normal fibroblasts (Fig 5.3) In contrast, mRNA expression of Fli-1 proto-oncogene (FLI1), a suppressor of collagen transcription, was significantly reduced in SSc fibroblasts, while expression levels of fibrillin 1 (FBN1) were not significantly different. Among samples cultured under vibratory conditions, expression levels of IL1A, IL4, FBN1, and LOX were significantly lower in SSc fibroblasts relative to 1X strain controls, while expression of IL6 and FLI1 was significantly increased.
Figure 5.3. Vibration attenuates the mRNA expression of scleroderma phenotypic markers on SSc fibroblasts. Relative mRNA expression levels of proinflammatory cytokines (interleukin 1, alpha 1 (IL1A1), IL4, and IL6), matrix remodeling-associated proteins including fibrillin-1 (FBN1), lysyl oxidase (LOX), and TIMP1), collagen-suppressing transcription factors (FLI1) relative to static control at day 7. \( n = 4 \) samples per group. \(* (P < 0.05)\) compared with normal dermal fibroblast group (Normal), \( \dagger (P < 0.05)\) compared with 1X Strain group in each cell. All bar graphs, mean ± SD.

5.4.4. Vibration negatively regulates the mRNA induction of scleroderma phenotypic targets by a fibrotic model in vitro

Cyclic strain was used as an in vitro fibrotic model to investigate the ability of vibration to counteract a profibrotic stimulus. SSc fibroblasts seeded in porous polyurethane sponges were exposed to one-time 15% cyclic tensioning (1X Strain), 1X Strain followed by 5% cyclic strain alone (Cyclic Strain) or in combination with vibration
Samples cultured under cyclic strain alone exhibited significant increases in profibrotic cytokines (TGFB1 and CTGF), the pro-inflammatory cytokine (IL6), and FBN1 relative to the 1X Strain control.

Figure 5.4. Vibration negatively regulates cyclic strain-induced mRNA expression of scleroderma phenotypic markers on SSc fibroblasts. Relative mRNA expression levels of scleroderma (SSc) fibrotic targets including 1) profibrotic cytokines, 2) TGF-β signaling moledules, 3) proinflammatory cytokines, and 4) key mediators involved in collagen crosslinking and accumulation. All data were normalized to static control at 7 days. n = 4 samples per group. *(P < 0.05) compared with 1X Strain group, †(P < 0.05) compared with cyclic strain group. All bar graphs, mean ± SD.

Samples exposed to Cyclic Strain + Vibration showed significant increases in the mRNA expression of CTGF, EDN1, and IL6, but significant reductions in TGFR2, IL4, FBN1, LOX, and TIMP1 relative to the Cyclic Strain alone group and significant reduction in IL1A relative to the 1X Strain control (Fig. 5.4). In addition, mRNA levels for SMAD7, a signaling inhibitor involved in TGF-β signaling; and FLI1, a transcription
mediator that negatively regulates collagen production by dermal fibroblast were significantly increased compared with cyclic strain or 1X Strain groups.

Figure 5.5. Vibration inhibits cyclic strain-induced collagen synthesis and accumulation and mechanical stiffening on SSc fibroblasts. Relative mRNA expression levels of fibrous collagens normalized to static control at 7 days (A), collagen content, substrate stiffness, and cell number (B). \( n = 4 \) samples per group. *\((P < 0.05)\) compared with 1X Strain group, †\((P < 0.05)\) compared with cyclic strain group; ns, nonsignificant. All bar graphs, mean ± SD.

5.4.5. Vibration inhibits cyclic strain-induced collagen synthesis and accumulation in Ssc fibroblasts

In order to examine the effect of vibration on collagen gene transcription, protein accumulation, and substrate stiffness, SSc fibroblasts seeded in porous polyurethane
sponges were cultured under one-time tensioning strain, cyclic strain, and cyclic strain in combination with vibration as described above. qRT-PCR results demonstrated that the mRNA expression levels of COL1A1 in SSc fibroblasts exposed to cyclic strain alone were significantly increased, although the difference was only statistically significant for COL1A1 (Fig. 5.5A). In contrast, samples cultured under cyclic strain in combination with vibration exhibited significant reductions in collagens COL1A1 and COL3A1 relative to both the cyclic strain and 1X strain control groups. In addition, cyclic strain produced significant increases in collagen accumulation and substrate elastic modulus relative to the 1X Strain control, while cyclic strain in combination with vibration significantly decreased collagen deposition and elastic modulus relative to both the 1X Strain and cyclic strain groups (Fig. 5.5B).

5.5. Discussion

Pathologic fibrosis can occur in every major organ, representing approximately 45% of all mortality worldwide [16,87,89]. Excessive deposition of extracellular matrix (ECM) components, particularly collagens is generally regarded as a major outcome of fibrotic diseases induced by a variety of stimuli including persistent scarring, chronic exposure to infections and cytokines, autoimmune and allergic reactions, and radio- and chemotherapy [151,392]. Myofibroblasts have long been regarded as a key effector cell in fibrogenesis and primary effector cell responsible for synthesis of collagen and other ECM components [77]. TGF-β, as the most important mediator is known to be up-regulated and activated in a wide range of fibrotic disease and modulate myofibroblast differentiation [24,143,393]. EDA-FN (extra domain A fibronectin) and mechanical
tension are also important factors in the differentiation of mature myofibroblasts and continuous exposure to these factors leads to increased stiffness of the fibrotic tissue and ultimately organ failure [40,394].

Many studies have shown that fibroblasts derived from clinically affected fibrotic lesions have altered mRNA expressions in cytokine and matrix-related genes that persist even over extended periods in culture. Recently, manipulation of fibrotic phenotype in myofibroblasts have gained considerable attention as a potential antifibrotic strategy. Several studies suggest that the profibrogenic phenotype of myofibroblasts can be controlled by substrate stiffness [111,165–168]. For example, Wang et al. demonstrated that valvular myofibroblasts grown on soft substrates (~ 7 kPa, mimicking healthy cardiac valve tissue) showed a decrease in α-smooth muscle actin (α-SMA) stress fibers and proliferation and an increase in apoptosis, while the mRNA expression levels of α-SMA and CTGF were significantly increased in valvular myofibroblasts cultured on stiff substrates (~ 32 kPa, mimicking pre-calcified diseased tissue) compared with cells grown on compliant substrates [165]. In addition, Goffin et al. reported that myofibroblasts cultured on three-dimensional culture substrates with an increased stiffness exhibited increased levels of α-SMA expression and induction of myofibroblast activation with TGF-β1 supplementation, however, when fibroblasts cultured on the substrates with a reduced tension corresponding to the compliance of normal connective tissue (e.g. skin), no increases in the expression of α-SMA protein and collagen matrices were observed by exogenous addition of TGF-β1 [395]. Moreover, Marinkovic et al. cultured IPF-derived fibroblasts in collagen hydrogel substrates with the stiffness spanning from normal and
fibrotic lung tissues [111]. They found that the contractile and proliferative function in fibrotic fibroblasts were significantly inhibited when they were cultured in soft matrices (~1 kPa of elastic modulus), approximating the physiological level of normal lung ECM stiffness, suggesting that the myofibroblast phenotype is not a permanent state but can be reversed by alterations in the matrix properties.

In addition to substrate stiffness, myofibroblast phenotype can be modulated by externally-applied mechanical stimuli. Significantly decreased mRNA levels of α-SMA and reduced cellular contractility were reported, when human peripapillary scleral myofibroblasts were subjected to 1% cyclic strain compared to 4% cyclic strain applied at 5 Hz [396,397]. The phenotypic conversion from myofibroblasts to fibroblasts can also be obtained by the exposure to continuous static stretching as indicated by significant decreases in collagen secretion and α-SMA mRNA expression [398–400]. Myofibroblast subjected to cyclic strain (5%, 1 Hz) exhibited reduced mRNA expression of myofibroblast markers (COL1A1, COL3A1, α-SMA, and TGF-β1) mediated by decreased phosphorylation of SMAD2, which is a downstream effector in TGF-β signaling [401]. Previously, we have shown that high frequency vibration down-regulated TGF-β signaling and inhibited the induction of collagen synthesis and mechanical stiffening in response to fibrotic stimuli. Here, using fibroblasts derived from both clinically involved and uninvolved skin regions of patients with SSc, we tested whether vibratory stimulation can reverse the profibrogenic phenotype in SSc fibroblasts.

A number of researchers have reported that SSc fibroblasts from clinically affected skin are known to exhibit numerous phenotypic differences with expression of
various cytokines and ECM components, particularly excessive expression of collagens (COL1A1 and COL3A1) [402]. As a key effector of TGF-β, CTGF plays important roles in the activation of SSc fibroblasts and promotes fibroblast proliferation and collagen deposition synergistically with TGF-βs. It is noted that CTGF was consistently up-regulated in the sclerotic lesion from patients with SSc [403]. In addition, CTGF mRNA expression levels are increased approximately six-fold in the lesional fibroblasts relative to nonlesional fibroblasts isolated from the same patients [404]. TGF-β receptor type 1 (TGFBR1) and TGFBR2 also exhibited a significantly increased expression in SSc fibroblasts compared with normal fibroblasts from health donors [379,405], while the mRNA levels of SMAD7, which is an endogenous inhibitor in TGF-β signaling, were significantly down-regulated in SSc fibroblasts [380]. Pro-inflammatory cytokines (IL1 [368] and IL6 [370]) and matrix remodeling-related proteins (TIMP1 [374], LOX [376], and FBN1 [406]) were significantly elevated in SSc fibroblasts from the affected lesion compared with those from unaffected lesion, while significant increases in IL4 mRNA levels in SSc fibroblasts were only investigated compared with normal fibroblasts [369]. In addition, the expression of FLI1 protein is consistently reduced in fibroblasts derived from clinically involved scleroderma skin, which leads to enhanced collagen synthesis in systemic sclerosis diseases [407,408].

We found that qRT-PCR results of lesional SSc fibroblasts versus nonlesional SSc fibroblasts on TGF-β-related genes were consistent with previous observations indicating differences in mRNA levels of major profibrogenic phenotypic targets in fibroblasts derived from the clinically affected lesion of patients with SSc compared with
nonlesional or normal fibroblasts. The present study confirms that vibration down-regulated TGF-β signaling through decreased expression of molecules involved in signal transduction including SMAD3 and TGF-β receptors (TGFBR2 and TGFBR3) and increased expression of molecules that are associated with signal inhibition (SMAD7, SIK1, and SKIL) relative to 1X Strain controls, demonstrating that vibration can still be effective in modulating the TGF-β responsiveness of fibrotic fibroblasts. Interestingly, significant increases in profibrotic cytokines (TGF-β, CTGF, and EDN1) were observed in response to vibration. In accordance with previous studies, it is widely reported that externally-applied mechanical stimulation induced increased expression of profibrotic cytokines and altered matrix synthesis and degradation [245,322,337,338,409,410]. Specifically, the expression of profibrotic cytokines such as TGFB1 and CTGF is mechanoresponsive and significantly increased in response to various mechanical stimulation by a number of cell and tissue types including lung fibroblasts [245,409], periodontal ligament fibroblast-like cell (PDL) [410], gingival fibroblasts [337], laryngeal fibroblasts [338], and tracheal fibroblast [322] compared to nonloaded condition. An increased expression of profibrotic cytokines by mechanical stimulation was known to promote collagen synthesis and accumulation. However, vibration significantly reduced collagen mRNA expression and synthesis, as well as mechanical stiffening of the substrates induced by cyclic strain through the down-regulation of TGF-β signaling.

In addition to alternations in TGF-β and collagen expression, fibroblasts derived from SSc lesional biopsies also exhibited alterations in a number of other targets
including inflammatory cytokines and molecules involved in matrix assembly, crosslinking, and stability in response to vibration. Our findings suggest that vibratory stimulation induced differential expression of the pro-fibrogenic markers through reduced expression of TGF-β receptor type 2 (TGFBR2), pro-inflammatory cytokines (IL1A and IL4), and matrix remodeling-associated molecules (FBN1, LOX, and TIMP1) and increased expression of SMAD7, an inhibitor in TGF-β signaling and FLI1, a suppressor of collagen transcription. Although many of profibrogenic phenotypic targets were activated in SSc fibroblasts as previous reported [92], vibration was able to positively control the pathologic markers of SSc fibroblasts.

Many studies suggest that mechanical stimuli including substrate stiffness and externally-applied mechanical forces play an important role in regulating the fibrotic phenotype and matrix expression [41,396–399]. As an in vitro fibrotic model, cyclic strain is a known profibrotic stimulus that promotes increased collagen deposition via a TGF-β-dependent mechanism [228,232,244]. When SSc fibroblasts were subjected to cyclic strain in combination with vibration, profibrogenic phenotype markers including molecules involved in collagen crosslinking processes (TGFBR2, IL4, FBN1, LOX, and TIMP1) were significantly decreased, while anti-fibrotic molecules (SMAD7 and FLI1, a collagen synthesis inhibitor) were significantly increased. Moreover, vibration exhibited significant reduction in COL1A1 mRNA levels induced by cyclic strain alone. Furthermore, strong induction in collagen content and increases in mechanical stiffening were significantly reduced in response to Cyclic Strain + Vibration relative to cyclic strain or 1X Strain control. These studies, therefore, suggest that profibrogenic phenotype
of scleroderma fibroblasts could be reversed by vibratory stimulation. Mechanical stimulation serves as an important mediator in the context of matrix remodeling, particularly collagen synthesis and degradation. As a novel antifibrotic mechanotherapy, vibration may offer several benefits relative to current anti-TGF-β strategies: 1) it can be easily and locally applied and 2) no systemic side effects can be triggered compared with systemically-administered pharmacological approaches. A limitation of present study was that scleroderma fibroblasts subjected to cyclic strain along with ascorbate supplementation were used as an in vitro fibrogenic model. However, fibrosis is a highly complex, dynamic disease and cannot be perfectly emulated in vitro. In addition, while we have mainly focused SSc fibroblasts as a key effector cell in fibrosis, it is important to acknowledge that a variety of inflammatory cells are involved in the fibrotic pathogenesis and the role of vibration on pro-inflammatory cytokines needs to be established in the future studies. In order to address this issue, the reversibility of profibrogenic phenotype mediated by vibratory stimulation will be further demonstrated in animal models with dermal fibrosis induced by gamma irradiation [411] or bleomycin [412].

5.6. Conclusion

This study is the first to show that high frequency vibration was able to reverse the profibrotic phenotype of SSc dermal fibroblasts by down-regulating TGF-β signaling, decreasing collagenous matrix remodeling-related genes (FBN1, LOX, and TIMP1), and increasing collagen transcriptional suppressors (FLI1). Moreover, vibration reduced the mRNA and protein expression of collagens COL1A1 and COL3A1 and decreased mechanical stiffness of polyurethane sponges seeded with SSc dermal fibroblasts,
suggesting that the myofibroblastic activation of SSc dermal fibroblasts could be reversed by vibration. Hence, vibration-mediated therapy could be used as a clinical approach for the treatment of a wide range of fibrotic diseases.
CHAPTER SIX

CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1. Conclusions

The overall goal of this dissertation was to investigate the roles of vibratory stimulation in the matrix remodeling in the superficial lamina propria of vocal folds and demonstrate the potential of vibratory stimulation for treatment of fibrotic diseases. Pharmaceutical approaches targeting particularly TGF-β and its downstream molecules have been regarded as promising strategies, however, no clinical treatments have successfully demonstrated their therapeutic effects against a wide range of fibrotic diseases. A small number of recent studies have shown that alteration of the mechanical microenvironment can alter matrix remodeling and reverse the pathogenic phenotype of myofibroblasts into quiescent fibroblasts. While these results are encouraging, their translational therapeutic application is challenging.

The present studies demonstrate for the first time the ability of externally applied mechanical stimulation in the form of high frequency vibration to inhibit fibrotic signaling, block increases in collagen and substrate stiffness in response to profibrotic stimuli, and reverse the fibrotic phenotype of fibroblasts derived from pathological tissue. It appears that the major mechanism is the ability of vibration to modulate the expression of a wide range of gene targets involved in TGF-β signaling, with the net result of reducing signaling capacity. However, these studies also demonstrate potential for accelerating catabolic remodeling of collagenous matrix, indicating that vibration may work through multiple mechanisms. The studies of gene expression in response to
variable vibratory stimulation demonstrate that while fibroblasts do exhibit dose and
temporally-dependent responses to vibration, many key targets such as HAS enzymes and
collagens respond similarly to a wide range of vibratory regimes, demonstrating the
robustness of this response. The observation that the changes in gene expression induced
by vibration are almost entirely reversed within 18 hours after removal of vibration
indicates that these are transient responses and that whatever final form vibratory
mechanotherapy may take, it will need to be designed in manner suitable for repeated
application. While these studies most strongly support the ability of vibration to inhibit
the initiation of fibrotic responses, studies demonstrating the capability of vibration to
promote turnover of previously synthesized matrix and alter expression of pro-
inflammatory genes suggest that vibration may also have potential to therapeutically
intervene at least in the early stages of disease progression.

6.2. Recommendations

The vibratory bioreactor used in these studies has been successfully manufactured
and used as described previously [231,322,325,365]. However, there are experimental
limitations that our vibratory module can only produce the frequency range from
approximately 25 to 150 Hz [413–416], which is close to the fundamental frequency
range in men’s voice. In future studies, the vibratory bioreactor that can provide more
broad range of frequency that corresponds to physiological levels (100-300 Hz) including
the fundamental frequency ranges of men and women will be needed to fully understand
the ECM regulation mechanism in response to vibration.
Moreover, numerous studies have demonstrated the crucial role of several fibrogenic cytokines released from macrophage and immunocytes in a variety of fibrotic diseases [53,87,417,418]. Thus, it is important to acknowledge that a variety of inflammatory cells are involved in fibrotic pathogenesis and the role of vibration on the expression of pro-inflammatory and anti-inflammatory cytokines needs to be established in the future studies. As a first step, fibroblast/macrophage co-culture system would be an appropriate model to investigate the effects of vibration on the key inflammatory components that are associated with fibrotic diseases.

Another important limitation of this research is that we have primarily focused on fibroblasts as the key effector cell of fibrotic diseases. However, fibrosis is a highly complex, dynamic, multicellular as well as progressive autoimmune disease that affects all major internal organs and eventually leads to organ dysfunction and failure [419]. Our *in vitro* models created by either exogenous addition of TGF-β1 or cyclic strain along with ascorbate supplementation cannot fully replicate the fibrosis model. The therapeutic efficacy of vibration, therefore, will be further demonstrated in the future studies using animal models with dermal fibrosis induced by gamma irradiation [411] or bleomycin [412].

However, a major challenge for the proposed *in vivo* studies is that our bioreactor module could not apply to the animal models due to its operational complexity and non-portability. Therefore, it will be necessary to design a small flexible, portable device suitable for delivering vibratory stimulation to test clinical efficacy of vibratory stimulation in animal models with fibrotic diseases. It is also essential to acknowledge
that SSc, keloids, and hypertrophic scars in the skin are the most readily accessible targets, however, substantial innovation and engineering design will be required for application to non-superficial tissues such as lung, liver, kidney, and heart. As an emerging technology, polymeric actuators are recently being investigated as a promising technology in biomedical applications such as microsensors and artificial muscles, owing to their flexibility, lightweight, performance, and ease of fabrication [420–424]. Specifically, the research conducted by Kim et al., showed that polymeric electroactive actuator composed of sulphonated block copolymers and ionic liquids produced a fast and robust millimeter-scale displacement at low voltages [425], suggesting a potential intervention that may be employed in the future studies. However, more thorough consideration in development of the vibration-producing devices will be required to achieve our long-term goal.
REFERENCES


[19] Roycik, M.D., Sang, X.F. and Q.-X., A Fresh Prospect of extracellular matrix


expression in fibroblasts is regulated by a three-dimensional contact with collagen.


[54] DiPietro, L.A., Polverini, P.J., Role of the macrophage in the positive and negative


[83] Gerber, H., Condorelli, F., Park, J., Ferrara, N., Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but


[113] Noble, P.W., Barkauskas, C.E., Jiang, D., Pulmonary fibrosis: patterns and


[125] Lipson, K.E., Wong, C., Teng, Y., Spong, S., CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. *Fibrogenesis Tissue Repair* 2012, 5, S24.


[141] Nagarajan, R.P., Zhang, J., Wei, L., Chen, Y., Regulation of Smad7 promoter by


[183] Huang, C., Holfeld, J., Schaden, W., Orgill, D., Ogawa, R., Mechanotherapy: Revisiting physical therapy and recruiting mechanobiology for a new era in


[222] Leung, D.Y., Glagov, S., Mathews, M.B., Cyclic stretching stimulates synthesis of


[274] Hirano, M., Kurita, S., Nakshima, T., in.; Stevens KN, Hirano M (Eds.), *Vocal


[283] Hahn, M.S., Kobler, J.B., Starcher, B.C., Zeitels, S.M., Langer, R., Quantitative and comparative studies of the vocal fold extracellular matrix. I: elastic fibers and


[301] Longaker, M.T., Chiu, E.S., Adzick, N.S., Stern, M., et al., Studies in fetal wound


[318] Gordon, K.J., Blobe, G.C., Role of transforming growth factor-β superfamily


[342] Watanabe, Y., Itoh, S., Goto, T., Ohnishi, E., et al., TMEPAI, a transmembrane TGF-β-inducible protein, sequesters Smad proteins from active participation in


