Availability of TGF-β1 in the Myofibroblast Matrix:

A Matter of Strain and Fibronectin

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Faculty of Dentistry
University of Toronto

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2014

Abstract

Fibrosis is a detrimental disease that causes organ failure with no effective therapy available to date. Activated fibroblasts – myofibroblasts – are responsible for the excessive secretion of extracellular matrix (ECM) and contraction into the stiff collagenous scar tissue that characterizes fibrosis. Transforming growth factor β-1 (TGF-β1) is the main cytokine that activates myofibroblasts from a variety of different precursor cells and is central regulator of all fibrotic processes. Because TGF-β1 also positively controls vascular, inflammatory, and epithelial cell homeostasis, global TGF-β1 inhibition strategies failed to treat fibrosis due to severe side-effects on other cell types.

My thesis aims to identify myofibroblastic-specific mechanisms of TGF-β1 activation as potential therapeutic targets to treat fibrosis. I am revealing how the mechanical and compositional properties of the ECM contribute to the extracellular storage and activation of latent TGF-β1 by myofibroblast contraction.
We are the first to establish a direct link between the ECM remodeling activity of myofibroblasts and TGF-β1 bio-availability. Analogous to Toronto squirrels storing chestnuts for the winter, myofibroblasts establish stores of latent TGF-β1 in the ECM for later activation to gain the contractile strength required to remodel scar tissue. My results demonstrate that: (1) Direct application of mechanical force to latent TGF-β1 through cell surface receptor integrins induces a conformational change in the TGF-β1-associated pro-peptide and releases the active growth factor. (2) Akin to loading a mechanical spring, myofibroblasts gradually strain the ECM that stores TGF-β1 and thereby mechanically prime the latent complex for more efficient release of active TGF-β1. (3) The presence of extracellular domain-A (ED-A), being characteristically spliced into cellular fibronectin in conditions of tissue remodeling, enhances binding of latent TGF-β1 to the myofibroblast-specific ECM.

In the course of these studies, I further show that inhibiting myofibroblast force production and integrin force transmission, blocking the binding of latent TGF-β1 to ED-A fibronectin, and releasing the myofibroblast ECM from strain all result in reduced amounts of active TGF-β1 being available to bind receptors in the cell membrane. Consequently, these interventions interrupt the feed-forward loop of myofibroblast activation by TGF-β1, enhanced contraction, and enhanced auto-activation of TGF-β1. Eventually, these findings are poised to lead to novel strategies to attenuate fibrosis progression in vivo using the myofibroblast and its ECM as specific target.
Acknowledgments

Firstly, I would like to thank my PhD supervisor, Dr. Boris Hinz, for the opportunity to conduct and complete my research project in his laboratory at the University of Toronto. I thank Dr. Christopher McCulloch for his support and all the suggestions that improved my research and for serving as my co-supervisor. Special thanks to Dr. Craig Simmons for being my third committee member and for providing valuable input for my research. Additionally, I thank Dr. Katalin Szaszi for serving as SGS member and Dr. Paul Janmey as external examiner on my final PhD oral defense.

I am grateful to all current and past Hinz Lab members as well as Matrix Dynamics Group students, staff and faculty members, for creating a stimulating and exciting environment for doing research. In particular, I want to thank, Dr. Anne Koehler for introducing me to the lab and managing the lab. I thank Melissa Chow for helping me out in protein purification and Nilesh Talele for critical scientific discussions. In addition, I would like to thank all the members of the Tissue Transmigration Training Network for their friendship and good-times during meetings.

Furthermore, I want to acknowledge the technical support provided by Dr. Lindsey Fiddes at the Center for Microfluidic Systems, Dr. Doug Holmyard at the Mount Sinai imaging facility and especially Cheung Lo for managing the cell culture facility with the highest degree of efficiency and providing excellent research conditions.

I thank for the funding support from the European Union's Seventh Framework Program (FP7/2007-2013) under grant agreement #237946 to Dr. Boris Hinz, the CIHR Cell Signals Training program and for the Harron Scholarship provided by the Faculty of Dentistry at the University of Toronto.

Finally, I want to thank all my friends and family for their role in maintaining my healthy work-life balance and I want to particularly thank my fiancée, Olga Luft, for her constant love and support during the course of this degree.
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List of Abbreviations

α-SMA – α-smooth muscle actin
μCP – micro contact printing
AFM – atomic force microscopy
AGE – advanced glycation end-products
BCA - bicinichoninic acid
BMP – bone morphogenetic protein
BSA – bovine serum albumin
CCN – cysteine rich 61, connective tissue growth factor, nephroblastoma overexpressed
CHO - Chinese hamster ovary
CTGF – connective tissue growth factor
DPBS - Dulbecco’s phosphate buffered saline
DMEM – Dulbecco’s modified eagles medium
DOC – desoxycholate
ECM – extracellular matrix
ED-A – extradomain A
ED-B – extradomain B
ELISA - enzyme linked immunosorbent assay
EMT – epithelial-to-mesenchymal transition
EndoMT – endothelial-to-mesenchymal transition
FA – focal adhesion
FAK – focal adhesion kinase
FGF – fibroblast growth factor
FITC - fluorescein isothiocyanate
FN – fibronectin
EGFP – enhanced green fluorescent protein
HA – hyaluronic acid
hDF – human dermal fibroblast
hDMf – human dermal myofibroblast
HEK – human embryonic kidney
HGF – hepatocyte growth factor
IMAC – ion metal affinity chromatography
KD – knock down
kPA – kilo Pascal
KO – knock out
LAP – latency associated peptide
LF – lung fibroblast
LLC – large latent complex
LOX - lysyl oxidase
LOXL - lysyl oxidase-like
LTBP-1 – latent TGF-β1 binding protein-1
MEF – mouse embryonic fibroblast
MMP – matrix metalloproteinase
WB – Western blot
PAGE - polyacrylamide gel electrophoresis
PAI – plasminogen activator inhibitor
PBS – posphate buffered saline
PDGF – platelet-derived growth factor
RGD – arginine-glycine-asparagine
SDS – sodium dodecyl sulfate
SEM – scanning electron microscopy
SLC – small latent complex
SLRP – small leucine-rich proteoglycans
siRNA – small interfering ribonucleic acid
SPARC - secreted protein acidic and rich in cysteine
TCP – tissue culture plastic
TG - transglutaminase
TGF-β1 – transforming growth factor-beta 1
TRAP-6 - thrombin receptor activator peptide-6
TRITC – tetramethylrhodamine isothiocyanate
TMLC – transformed mink lung epithelial cell
VEGF – vascular endothelial growth factor
WISP - Wnt-1 induced secreted protein 1
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Chapter 1 – Background

1.1 Rationale

Organ fibrosis is a major cause for death following heart, lung, liver, kidney, and skin diseases (Hinz and Gabbiani, 2010; Wynn and Ramalingam, 2012). Transforming growth factor-β1 (TGF-β1) is the most potent pro-fibrotic cytokine known; it causes excessive production of extracellular matrix (ECM), induces its own secretion and drives differentiation of various precursor cells into myofibroblasts (Grainger, 2007; Hinz, 2007; Leask and Abraham, 2004; Ruiz-Ortega et al., 2007). Myofibroblasts are the main culprits for the excessive production of collagenous ECM and detrimental tissue contractures characteristic for fibrosis. Their excessive contractile activity is mediated by expression of α-smooth muscle actin (α-SMA) in stress fibres. Both, myofibroblasts and TGF-β1 have been identified as main targets to attenuate fibroproliferative diseases such as fibrosis and cancer (Van De Water et al., 2013).

Three factors are pivotal for myofibroblast differentiation and function: TGF-β1, a mechanically resistant ECM, and the fibronectin (FN) splice variant extradomain-A (ED-A) FN (Hinz, 2009). Current pharmaceutical and translational approaches that have entered clinical trials mostly target the active form of TGF-β1 (Howell and McAnulty, 2006; Liu et al., 2006; Meier and Nanney, 2006; Varga and Pasche, 2008). However, none of these strategies have been successfully implicated in resolving the persistent nature of fibrosis (Varga and Pasche, 2008). The limitation of such global strategies is the interference with beneficial effects of the pleiotrophic TGF-β1, such as controlling homeostasis of epithelial, vascular, endothelial, and of immune cells (Jenkins, 2008; ten Dijke and Arthur, 2007; Wakefield and Stuelten, 2007).
Figure 1: TGF-β1 activation by integrin pulling.

Latent TGF-β1 is stored in the ECM by association with LTBP-1. Interaction of integrins with LAP activates TGF-β1 upon myofibroblast contraction by a putative conformation change in the LLC (Hinz, 2009).

Another possibility is to prevent TGF-β1 activation in a cell type-specific manner rather than blocking the already active TGF-β1. Myofibroblasts secrete TGF-β1 together with its latency-associated pro-peptide LAP. Association of this small latent complex (SLC) with the latent TGF-β1 binding protein-1 (LTBP-1) provides a reservoir of latent TGF-β1 in the ECM by binding to FN (Figure 1). Different cell types use different mechanisms to activate latent TGF-β1 from this large latent complex (LLC) (Annes et al., 2004a; Jenkins, 2008; Sheppard, 2005; Wipff and Hinz, 2008).

The recent discovery of our lab of the cellular mechanisms of TGF-β1 activation by lung myofibroblasts (Wipff et al., 2007) provides a new framework for possible therapeutic interventions. Myofibroblasts, literally, pull on the LLC to activate TGF-β1 (Wipff et al., 2007).
The pulling force is directly transmitted to the LLC via transmembrane integrins, leading to a conformational change in LAP and release of the active TGF-β1 (addressed in Chapter 2) (Figure 1). Our lab has further suggested that TGF-β1 activation by integrin pulling requires anchorage of latent TGFβ1 to a sufficiently stiff ECM (Wipff et al., 2007) (Figure 2). In my thesis project, we propose that myofibroblast-generated strain ‘primes’ the LLC-containing ECM for effective TGF-β1 release - analogous to loading a spring (addressed in Chapter 3).

Figure 2: A schematic view on latent TGFβ1 activation by cell traction.
Integrin binding to a specific RGD site in the LAP portion transmits intracellular force to the latent TGFβ1 complex. In the context of a soft ECM (unloaded springs) cell pulling will simply drag the latent complex. When bound to a remodeled stiff ECM (loaded springs) integrin-mediated force exertion can trigger a conformation change in the LAP and make TGFβ1 available for its receptor (Hinz, 2010b).
The ED-A-containing splice variant of FN has been identified as another pivotal element in myofibroblast differentiation, but the mechanism of its action is virtually unknown (Serini et al., 1998a) (Figure 3). ED-A FN is neo-expressed in healing wounds and up-regulated in organ fibrosis (Muro et al., 2008; Serini and Gabbiani, 1996). Our lab has previously shown that mechanical stress increases ED-A FN expression in wound granulation tissue (Hinz et al., 2001a). Blocking the ED-A domain with specific antibodies inhibits myofibroblast differentiation (Serini et al., 1998a). ED-A FN deficient mice are protected from experimentally induced lung fibrosis, and exhibit significantly reduced numbers of myofibroblasts (Muro et al., 2008). For unknown reasons, ED-A-null mice fail to develop lung fibrosis and present a diminished capacity for activation of TGF-β1 (Muro et al., 2008). We propose that ED-A FN exhibits specific characteristics in interacting with LTBP-1. This is not unreasonable as FN is a major binding partner of LTBP-1 in the ECM of fibroblasts (Unsold et al., 2001). However, neither the FN splice variant specificity nor the influence of mechanical strain have been assessed in this interaction and will be addressed in Chapter 4 of this thesis.

**Figure 3: Scheme of the FN domain structure.**

Shown is one arm of the FN dimer. Each FN subunit consists of three repeats: Type I, II and III. The first five Type I repeats are required for FN assembly. Repeats 6-11 mediate binding to collagen or gelatin. The three Type III repeats (12-14) are required for binding to heparin and syndecans. Cellular FN contains ED-A (red) and/or ED-B (purple) domain whereas soluble plasma FN lack ED-B and ED-A. Between repeat 14 and 15 is a variable region (green).
1.2 Significance and Disease Context

This thesis is situated in the context of skin scarring and fibrosis. Skin connective tissue contractures are a major clinical problem. Severe conditions include hypertrophic scarring following burn wounds and fibrosis affecting systemic sclerosis (scleroderma), fibromatosis and host-implant contractures (Atiyeh et al., 2005; Falanga, 2005). Scarring is far more than an aesthetic problem; the stiff fibrotic scar reduces functionality of the affected skin area and hinders free movement of limbs. The detrimental contractures and stiffening of connective tissue observed in skin fibrosis are generated by myofibroblasts. Importantly, the increasing tissue stress produced by the myofibroblast feeds back on this mechano-responsive cell and leads to continued accumulation of scar tissue. Our research aims at interrupting this viscous loop by interfering with the mechanical activation of pro-fibrotic TGF-β1 from the myofibroblast ECM.

Normal dermis contains high levels of LTBP-1, potentially acting as a TGF-β1 repository (Karonen et al., 1997; Raghunath et al., 1998). The levels of total TGF-β1 are elevated during all stages of wound healing (Brunner and Blakytny, 2004). Active TGF-β1 however only peaks transiently during early inflammation and persistently during the later phase of wound contraction by myofibroblasts (Yang et al., 1999). The question arises why α-SMA-negative fibroblasts, which are abundant during early wound remodeling, do not activate latent TGF-β1 deposits from the ECM. Previously, our lab suggested that the ECM of early wounds is too soft to support traction-mediated TGF-β1 and that fibroblasts are not sufficiently contractile to liberate TGF-β1 from the LLC by traction. New data produced in the framework of my thesis however indicates that this view is too simplified. In fact, the efficacy of force-mediated TGF-β1 activation depends on the mechanical and protein composition properties of the myofibroblast ECM that are established before the actual TGF-β1 activation step occurs.
1.3 Hypothesis

I hypothesize that the efficacy of latent TGF-β1 activation by myofibroblast contraction increases with increasing pre-strain in the ECM and is supported by binding of latent TGF-β1 to the ‘fibrotic’ FN splice variant ED-A FN.

1.4 Objectives and Specific Aims

Objective 1: To establish whether direct application of force to the LAP portion of LLC is sufficient to release active TGF-β1.

If the process of TGF-β1 activation by myofibroblast integrins is purely mechanical it must be possible to achieve TGF-β1 activation in a cell-free system, using a pre-assembled ECM containing the latent TGF-β1 complex and experimental pulling forces transmitted to latent TGF-β1 through purified integrins.

Specific Aim 1.1: To mechanically activate TGF-β1 in a completely cell-free system and measure TGF-β1 release from the LLC.

Objective 2: To test whether myofibroblast remodelling and pre-straining of the ECM increases the availability of TGF-β1 for subsequent activation by mechanical forces.

Mechanical activation of TGF-β1 from a mechanically resistant ECM is currently understood as an acute process that depends on the contractile force of cells. We propose that ECM remodeling, preceding the activation step, mechanically primes latent TGF-β1 akin to loading a mechanical spring. This mechanism would ultimately support the concept of myofibroblast auto-stimulation in the progression of fibrosis.
Specific Aim 2.1: To test whether and how expression and fibrillar organization of LTBP-1 and ED-A FN change over time during experimentally induced myofibroblast differentiation in vitro and in vivo.

Specific Aim 2.2: To compare low contractile fibroblasts and highly contractile myofibroblasts for their capacity to promote LTBP-1 fibrillogenesis.

Specific Aim 2.3: To decipher the roles of FN, fibrillin-1, and specific integrins to aid organization of LTBP-1 in the myofibroblast ECM.

Specific Aim 2.4: To demonstrate a direct link between the level of pre-strain in the myofibroblast ECM and the efficacy of TGF-β1 activation by mechanical force in cell-free and cell-containing in vitro conditions.

Objective 3: To decipher how the ED-A domain in FN contributes to LTBP-1 binding

The ED-A-containing splice variant of FN is a pivotal element in myofibroblast differentiation, but the mechanism of its action is unclear. We propose that ED-A FN exhibits specific characteristics in interacting with LTBP-1 compared to other FN splice variants and that this interaction facilitates storage of LTBP-1 in the myofibroblast ECM.

Specific Aim 3.1: To test whether ED-A FN and LTBP-1 expression and colocalization in the myofibroblast ECM are controlled by cell substrate stiffness.

Specific Aim 3.2: To test whether the ED-A domain of FN is essential or supportive for LTBP-1 binding to FN and incorporation into the ECM.
1.5 Overview of Chapters and Published Manuscripts

My thesis establishes an important interplay of TGF-β1, myofibroblasts and the ECM and establishes a novel role of myofibroblasts in mechanically priming the TGF-β1 complex for subsequent activation. Furthermore, I discover a new role of the ED-A domain in FN in binding the TGF-β1 storage protein LTBP-1. To investigate how ECM remodelling by myofibroblasts and the presence of ED-A FN in the myofibroblast ECM contribute to the bioavailability of TGF-β1, we are using primary human dermal fibroblasts (hDF) as our main cell model. During my thesis, I established a number of sophisticated *in vitro* models to address the mechanistic aspects of latent TGF-β1 ECM incorporation and subsequent activation by myofibroblast contraction. This includes cloning, production and purification of recombinant components of the LLC, establishing decellularized ECM, a variety of mechanical assays to strain ECM, cells, and single molecule complexes, and the use of cell contraction agonists to liberate TGF-β1 from the myofibroblast ECM.

*Chapter 1* has been published in the Journal of Pathology in 2013 as literature review that focuses on the detrimental functions of myofibroblasts, in particular, the excessive secretion of ECM (Klingberg et al., 2013). Serving as thesis introduction, this manuscript provides an overview on the main ECM components secreted by myofibroblasts and their association with fibrosis. Mutations and modifications of ECM components can cause severe disease manifestations. Additionally, the remodeling activity of myofibroblast can lead to tissue alterations or destruction, if deregulated. Here, I introduce the superfamily of FNs, fibrillins and LTBPs providing background information on isoforms, protein structure and disease associations required in the following chapters.
In Chapter 2, I am presenting a novel assay to activate TGF-β1 from decellularized ECM using ferro-magnetic beads coated with latent TGF-β1-binding integrins or antibodies. This assay was an essential part in the work of a study published in Current Biology in 2011 (Buscemi et al., 2011b). In this study, we employed atomic force microscopy to identify the amount of force needed to release a single molecule of TGF-β1 from the latent complex. Furthermore, we found that this process is an “all-or-nothing” mechanism at the single-molecule level. My experiments were essential to show that binding of integrins to the latent complex and the application of a pulling force leads to the release of measurable TGF-β1. The assays and protein constructs developed in the course of this study were essential to investigate different levels of ECM organization in my following work.

Chapter 3 represents the main part of my PhD project, which is in press with the Journal of Cell Biology (Klingberg et al., 2014). I show that myofibroblasts mechanically prime the latent TGF-β1 complex for efficient TGF-β1 activation. One of the key findings is that fibroblasts and myofibroblasts are different in their ability to organize the ECM. Myofibroblasts organize LTBP-1 into denser and substantially more strained fibrils than fibroblasts. Because LTBP-1 links the latent TGF-β1 to the ECM, fibroblasts tune active TGF-β1 bio-availability already during ECM deposition. In order to mimic cell mediated fibril strain, I used a highly expandable silicone substrate in combination with a unique mechanical strain device. I could show that with increasing fibril strain, TGF-β1 is released more readily. Additionally, I provide first evidence that human dermal fibroblasts bind to LTBP-1 directly using RGD-binding integrin receptors, such as αvβ1 and αvβ3.
Chapter 4 builds upon a manuscript that is ready for submission to Molecular Biology of the Cell. Since I have discovered that FN is essential for LTBP-1 assembly and the priming process of the latent TGF-β1 complex; I now set out to study the specific interaction of LTBP-1 with the myofibroblast-characteristic ED-A domain in the FN molecule. This work focuses on the importance of ED-A fibronectin in storing TGF-β1 via the binding of LTBP-1. In previous literature, the ED-A domain in fibronectin was blocked using antibodies. Blocking the ED-A domain in fibronectin lead to reduced myofibroblast differentiation and α-SMA expression. In order to extend our knowledge of LTBP-1 and fibronectin protein-protein interaction, I produced and overexpressed fibronectin domain peptides, full length fibronectin proteins lacking ED-A and/or ED-B domains, and down-regulated fibronectin in hDF and MRC-5 cultures. My results show that the presence of the ED-A domain in fibronectin enhances LTBP-1 deposition and absence reduces LTBP-1 incorporation and colocalization.

Chapter 5 summarizes my thesis with a discussion that elaborates that cells have the ability to control TGF-β1 activity by the way they organize LTBP-1 fibrils. ECM fibril strain determines the threshold that contractility of cell needs to overcome to release TGF-β1. In addition, the amount of LTBP-1 bound to the ECM is regulated by the expression of major ECM proteins such as FN or fibrillin and their respective splice forms and domain structures. The combination of ECM composition, e.g. amount of ED-A FN, and fibril structure contributes the TGF-β1 biology and availability.
1.6 Introduction - The Myofibroblast Matrix: Implications for Tissue Repair and Fibrosis

The contents of this section have been published in:

Journal of Pathology, 2013 January; 229(2):298-309

“The Myofibroblast Matrix: Implications for Tissue Repair and Fibrosis”

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My contribution to this work as first author has been to summarize the current knowledge of major myofibroblast ECM components such as collagens, FNs, fibrillins, and LTBPs. The highly synthetic phenotype of the myofibroblast creates a very unique composition and structure of the ECM. During wound healing this provisional ECM is needed to fill the void after injury but in pathological conditions, the myofibroblast ECM provides an environment favoring fibrosis development. This chapter introduces the key players that influence myofibroblast activation and persistence, and highlights their role in modifying TGF-\(\beta\) biology.
1.6.1 Abstract

Myofibroblasts, and the ECM in which they reside, are critical components of wound healing and fibrosis. The ECM, traditionally viewed as the structural elements within which cells reside, is actually a functional tissue whose components possess not only scaffolding characteristics, but also growth factor, mitogenic, and other bioactive properties. Although it has been suggested that tissue fibrosis simply reflects an ‘exuberant’ wound-healing response, examination of the ECM and the roles of myofibroblasts during fibrogenesis instead suggest that the organism may be attempting to recapitulate developmental programs designed to regenerate functional tissue. Evidence of this is provided by the temporospatial re-emergence of embryonic ECM proteins by fibroblasts and myofibroblasts that induce cellular programmatic responses intended to produce a functional tissue.

In the setting of wound healing (or physiologic fibrosis), this occurs in a highly regulated and exquisitely choreographed fashion which results in cessation of haemorrhage, restoration of barrier integrity, and re-establishment of tissue function. However pathologic tissue fibrosis, which oftentimes causes organ dysfunction and significant morbidity or mortality, likely results from dysregulation of normal wound healing processes or abnormalities of the process itself. This review will focus on the myofibroblast ECM and its role in both physiologic and pathologic fibrosis, and will discuss the potential for therapeutically targeting ECM proteins for treatment of fibrotic disorders.
1.6.2 Background

Myofibroblast activation is a key event in physiological and pathological tissue repair. Myofibroblasts are the primary ECM-secreting cells during wound healing and fibrosis, and are largely responsible for the contraction of scar tissue as it matures (Hinz et al., 2012; Wynn, 2008). The contribution of myofibroblasts and their elaborated ECM to normal and pathologic tissue repair has been well-studied in lung (Araya and Nishimura, 2010; Hardie et al., 2009; Wynn, 2011), liver (Hernandez-Gea and Friedman, 2011; Iredale, 2008; Iwaisako et al., 2012), kidney (Grande and Lopez-Novoa, 2009; Meran and Steadman, 2011), skeletal muscle (Serrano et al., 2011), systemic sclerosis (Asano, 2010b; Beyer et al., 2012; Varga and Abraham, 2007), heart (Krenning et al., 2010; Rohr, 2009; van den Borne et al., 2010), and the stromal reaction to tumours (De Wever et al., 2008; Otranto et al., 2012).

A number of recent reviews have considered the nature of myofibroblast progenitors in different organs (Hinz et al., 2007), including resident fibroblasts (Cirri and Chiarugi, 2011; De Wever et al., 2008; Desmoulière, 2007; Dranoff and Wells, 2010; Hinz, 2007; Porter and Turner, 2009), fibrocytes (Bellini and Mattoli, 2007; Herzog and Bucala, 2010; Keeley et al., 2011), smooth muscle cells (Coen et al., 2011), pericytes (Armulik et al., 2011; Humphreys et al., 2010; Kidd et al., 2012; Lin et al., 2008), epithelial and endothelial cells undergoing endothelial (EndoMT) or epithelial-to-mesenchymal transition (EMT) (Burns and Thomas, 2010; Chapman, 2011; Lee and Nelson, 2012; Piera-Velazquez et al., 2011; Quaggin and Kapus, 2011), mesenchymal stromal cells (Hinz, 2010a; Mishra et al., 2009), and hepatic stellate cells (Desmoulière, 2007), to name only the most prominent.
Others focus on the chemical and mechanical conditions controlling myofibroblast formation and survival (Kis et al., 2011), functional and phenotypic characteristics (Follonier Castella et al., 2010b; Sandbo and Dulin, 2011), and their suitability as therapeutic targets (Douglass et al., 2008; Hinz and Gabbiani, 2010; Leask, 2010; Scotton and Chambers, 2007; Sivakumar et al., 2012). Due to space constraints, these concepts will not be explored here.

Despite the abundant literature concerning the myofibroblast, surprisingly little focuses on specific features and functions of the myofibroblast ECM. Indeed, disturbance of the ECM and remodelling by myofibroblasts has profound impact on their own behaviour and that of other cell types sharing the same microenvironment. This is intuitive since the ECM performs a multitude of biological functions, including providing mechanical stability, protection and guidance for cells (Frantz et al., 2010; Hynes, 2012; Ozbek et al., 2010) and acting as a repository for growth factors (Discher et al., 2009b; Macri et al., 2007; Schultz and Wysocki, 2009).

1.6.3 The myofibroblast: born to produce and remodel ECM

Myofibroblasts were first identified four decades ago as fibroblastic cells that simultaneously exhibit prominent endoplasmic reticulum and contractile microfilament bundles in wound granulation tissue (Gabbiani et al., 1971). One prominent feature of the myofibroblast is the neo-expression of α-smooth muscle actin (α-SMA) in stress fibres (Tomasek et al., 2002), the molecular basis for the high contractile activity. However, not all α-SMA-expressing cells are myofibroblasts.
For example, α-SMA-positive cells that do not form microfilament bundles are not considered myofibroblasts since they are lacking their defining contractile element (Hinz, 2010b). Conversely, α-SMA-negative fibroblasts that express microfilament bundles are functional contractile myofibroblasts, at least in vivo. Since fibroblasts almost inevitably form microfilament bundles (stress fibres) in standard cell culture conditions, ‘myofibroblast’ denotes α-SMA-positive stress fibre-forming cells.

It bears mentioning that much of our understanding of myofibroblast behaviour arises from in vitro studies in which culture conditions vary greatly (e.g. culturing in ECM-coated dishes, culturing on “soft” agar, culturing in attached or detached collagen gels). This may account for discrepant results among studies and should be considered when interpreting data reported in the literature. One must also recognize that in vivo, fibroblasts and myofibroblasts encounter multiple ECM components simultaneously, thereby potentially altering behaviours from those observed in the experimental setting. Certainly, differences between fibroblast behaviour in 2-dimensional and 3-dimensional culture conditions are well-documented (Cukierman et al., 2001) and also inject a variability into results of in vitro studies. Finally, the role of mechanotransduction – the sensing of matrix stiffness and response to such stiffness by cells – is beginning to be elucidated in fibroblasts and myofibroblasts, and also adds yet another layer of complexity to our understanding of the myofibroblast ECM. Indeed, a percentage of fibroblasts spontaneously acquire a myofibroblast phenotype in culture (Hinz, 2010b; Rohr, 2011), likely due to stiffness of the culture vessel.
Figure 4: The myofibroblast ECM.

Schematic of some of the ECM molecules relevant to tissue fibrosis. The myofibroblast (center, with red stress fibres containing α-smooth muscle actin) lies enmeshed in its ECM (green). Components of the ECM are depicted (clockwise, from the 12 o'clock position): elastins, fibrillins and LTBPs, proteoglycans, tenascins, matricellular proteins, collagens, and fibronectins. the myofibroblast encounters, signals, and modulates the expression of these various components as outlined in the text.

Although collagen I and III are often cited as the primary ECM proteins expressed from myofibroblasts, the myofibroblast produces myriad other ECM proteins during wound repair and fibrosis. Myofibroblasts are capable of producing several ECM components, such as collagens type I, III, IV and V (Zhang et al., 1994), glycoproteins, and proteoglycans such as fibronectin, laminin, and tenasin (Aigner et al., 1997; Akhmetshina et al., 2008; Berndt et al., 1994; Chang et al., 1988; Crabb et al., 2006; Hinz, 2007; Kuhn and McDonald, 1991; Magro et al., 1997; Mahida et al., 1997; Stokes et al., 2001; Tomasek et al., 1986; Whiting et al., 2003; Zwetsloot et al., 2012).
It is worth noting, however, that myofibroblasts are not necessarily the only source of these proteins, as epithelial, inflammatory, and endothelial cells may all produce these proteins as well. A schematic of the myofibroblast ECM to be discussed is shown in Figure 4.

### 1.6.4 Collagens

Collagens are primarily structural proteins composed of three procollagen chains configured in a classic triple helical pattern. Early in the course of wound granulation, myofibroblasts deposit type III collagen. This form imparts a measure of plasticity to the wound in the early phase of healing, although recent data suggests that collagen III deficiency promotes myofibroblast differentiation and wound contraction (Volk et al., 2011). When granulation tissue is resorbed following physiologic wound repair, myofibroblasts undergo apoptosis (see below) and the more rigid type I collagen is biochemically identified. Under pathologic conditions (e.g. the proliferative cellular phase of palmar fibromatosis or areas of mesenchymal stromal invasion in breast carcinomas) type III collagens appear to be increased (Lagacé et al., 1985; Schürch et al., 1982), as are type V collagens in desmoplastic human breast carcinomas and in small airway fibrosis of bronchiolitis obliterans complicating chronic lung transplant rejection (Barsky et al., 1982; Mares et al., 2000; Wilkes et al., 2001). Of course, densely fibrotic tissues demonstrate an abundance of Type I collagens, but also Type VI collagens (Betz et al., 1993; Mollnau et al., 1995; Sabatelli et al., 2012; Specks et al., 1995; Zeichen et al., 1999).
As wound healing approaches completion, apoptotic gene programs are expressed within myofibroblasts, resulting in a relatively hypocellular scar. Cytokines that stimulate extracellular matrix synthesis early on are repressed once wound closure is completed and a functional basement membrane has been synthesized, thus suggesting the existence of a feedback loop (Streuli et al., 1993). However, in pathologic fibrosis, evidence suggests failure to initiate apoptosis of myofibroblasts (or decreased sensitivity to apoptotic stimuli) accounts for the seeming persistence of these cells in fibrotic tissues.

1.6.5 Fibronectins (FNs)

FN is expressed by multiple cell types and plays a key role in cell adhesive and migratory behaviour (Hynes, 1990; White et al., 2008). The functional FN dimer consists of two similar or identical subunits of 220–250 kDa that are held together by two disulphide bonds near their carboxyl-termini. Like many glycoproteins, each monomer is comprised of a combination of different types of homologous repeating domains; in the case of FN there are three, termed Types I, II and III. However, by virtue of alternative splicing of the pre-mRNA, two extra Type III repeats (termed ED-A for extra domain A and ED-B for extra domain B) may be inserted into the mature protein; the splicing of these domains are independent of each other (Muro et al., 1998; White et al., 2008) but are highly upregulated by the pro-fibrotic cytokine TGF-β1 (Borsi et al., 1990).

Alternative splicing of FN is particularly prominent during embryonic development, as well as during wound healing, pathologic fibrosis and malignancy, and gives rise to the term "oncofetal" ECM.
Evidence suggests that alternatively-spliced ED-A FN (but not plasma FN) is necessary for TGF-β1-induced myofibroblast differentiation (Kohan et al., 2010; Serini et al., 1998a) and is thus a critical component of the myofibroblast ECM.

As a separate contribution, FN also binds a large number of growth factors that may promote myofibroblast differentiation. Most notably, FN localizes latent TGF-β1 complex by binding latent TGF-β binding proteins (LTBPs, see below) (Fontana et al., 2005). In addition, FN binds vascular endothelial growth factor (VEGF) (Wijelath et al., 2006), bone morphogenetic protein (BMP) 1 (Huang et al., 2009a), hepatocyte growth factor (HGF) (Rahman et al., 2005), fibroblast growth factor (FGF)-2 (Bossard et al., 2004), and platelet-derived growth factor (PDGF) (Smith et al., 2009), all of which may contribute to the myofibroblast phenotype.

1.6.6 Elastin

Elastin, a major ECM protein involved in connective tissue homeostasis, provides organs with structural integrity and is responsible for absorption of mechanical overload preventing damage (Baldock et al., 2011). Smooth muscle cells and fibroblasts are the major elastin producing cells in normal tissues (Uitto et al., 1991) such as skin, heart, arteries, and lung, which all undergo cyclic mechanical loading and unloading throughout life. Elastin deposition and organization occurs mainly during the late foetal and early neonatal periods and is reduced during maturity to a low turn-over rate (Swee et al., 1995).
It has been generally understood that elastin production by fibroblasts is low or absent following injury which partly accounts for the reduced elasticity and breaking strength of scar tissue compared with the intact connective tissue (Schultz and Wysocki, 2009). Because of the low elastin turnover in normal and injured skin and arteries, current strategies aim in supplying elastin-like proteins either by grafting (Annabi et al., 2010; Caves et al., 2011; Lamme et al., 1996) or by stimulating cellular elastin production (Dong and Majesky, 2012; Venkataraman and Ramamurthi, 2011). However, some studies have shown that elastin production by fibroblasts is quite elevated after tissue damage in response to a number of cytokines, such as TNF-α, IL-1β and TGF-β1 (Pierce et al., 2006; Rosenbloom et al., 1991). For example, in constrictive bronchiolitis obliterans, characterized by fibrosis development in the small airways, α-SMA positive myofibroblasts demonstrated enhanced elastin expression (Shifren et al., 2007).

1.6.7 Fibrillins and LTBPs

In addition to the major fibrillar components, the myofibroblast ECM contains a microfibrillar network formed by members of the fibrillin and latent TGF-β binding protein (LTBP) family. In humans, these glycoprotein families consist of three homologous fibrillin isoforms (fibrillin-1, -2 and -3) and four LTBPs (LTBP-1, -2, -3 and -4) that are mainly characterized by highly repetitive and disulphide–rich domains.

Microfibrils provide the basis for tropoelastin binding during elastic fibre formation, enhance structural integrity of tissues and organs and target growth factors such as TGF-β and bone morphogenic protein (BMP) to the ECM (Ramirez and Rifkin, 2009; Ramirez and Sakai, 2010; Wagenseil and Mecham, 2007).
The disruption of microfibrillar assembly or growth factor association with fibrillins due to mutations within fibrillin genes lead to clinically relevant pathological connective tissue conditions such as Marfan’s syndrome, congenital contractual arachnodactyly, and systemic scleroderma (Avvedimento and Gabrielli, 2010; Barisic-Dujmovic et al., 2007; Fleischmajer et al., 1991; Godfrey, 1993; Ramirez and Dietz, 2007). LTBPs share similarities with fibrillins in their repetitive sequence and domain structure. However, LTBPs are considerably smaller, ranging from 125 kDa to 160 kDa when compared to fibrillins (~350 kDa). Analysis of LTBP isoforms from cultured human hepatic myofibroblast ECM reveals all four isotypes, suggesting these proteins may play a role in liver fibrosis (Mangasser-Stephan et al., 2001). Moreover, culture studies may give an insight of the sequence of events in ECM assembly by fibroblasts during embryogenesis and tissue repair. Recent mouse fibrillin-1 knock-out studies showed that LTBP-1 incorporation into the ECM of fibroblasts depends on a FN network as compared to the ECM association of LTBP-3 and LTBP-4 that is depended on fibrillin-1 microfibrils (Zilberberg et al., 2012b). In fibroblasts that are missing the gene for FN, LTBP-1 fails to incorporate into the ECM in the early phase but can be assembled in later stages (Dallas et al., 2005). LTBP-1 is crucial for tissue repair, fibrosis and myofibroblast biology because it serves as storage protein for TGF-β1. The TGF-β family comprises multipotent cytokines modulating cell growth, apoptosis, inflammation, and ECM synthesis. In mammals, these functions are mediated by the widely expressed three isoforms TGF-β1, TGF-β2 and TGF-β3 that are encoded by three different genes of high homology (Annes et al., 2003). TGF-β1 appears to be the most prevalent isoform that associates with fibroblast-to-myofibroblast activation (Desmoulière et al., 1993) although both other
isoforms have also been demonstrated to perform this action \textit{in vitro} (Serini and Gabbiani, 1996). \textit{In vivo}, TGF-β1 appears to attain a myofibroblast-suppressing role (Serini and Gabbiani, 1996; Shah et al., 1995). LTBP-1 regulates the bioactivity of TGF-β1 at multiple levels: 1) It promotes efficient latent TGF-β1 secretion by assembling the large latent complex (Yoshinaga et al., 2008), 2) targets latent TGF-β1 as a large latent complex to the ECM by interacting with different proteins including FN and fibrillin (Hynes, 2009; Ramirez and Rifkin, 2009; Todorovic and Rifkin, 2012), and 3) it controls and directs cell-mediated TGF-β1 activation (Jenkins, 2008; Todorovic and Rifkin, 2012; Wipff and Hinz, 2008). In addition to the aforementioned, myriad other ECM components can be found in the myofibroblast ECM, including fibulins, matricellular proteins (such as CCN proteins, osteopontin, periostin, and SPARC to name but a few), tenascins, and thrombospondins. These proteins have all been implicated in fibrogenesis and wound repair to various degrees, with the matricellular protein CCN2 (connective tissue growth factor, CTGF) and tenascin-C perhaps being the best studied. Similarly, experimental data supporting the role of WISP-1, SPARC, osteopontin, and thrombospondins in myofibroblast functions in wound healing and fibrosis have been amply documented (Borkham-Kamphorst et al., 2012; Bradshaw, 2009; Colston et al., 2007; Crawford et al., 1998; Frangogiannis, 2012; Grotendorst and Duncan, 2005; Harris et al., 2010; Heise et al., 2011; Kos and Wilding, 2010; Lenga et al., 2008; McCurdy et al., 2010; McCurdy et al., 2011; Mori et al., 2008; Rentz et al., 2007; Schultz and Wysocki, 2009; Singh et al., 2010a; Sweetwyne and Murphy-Ullrich, 2012). Below, we will highlight some recent evidence of the roles of these ECM proteins in wound repair and fibrosis.
1.6.8 CCN2 (CTGF)

CCN proteins (so named because of the names of the first three family members identified: cysteine rich 61 (CYR61), connective tissue growth factor (CTGF), and nephroblastoma overexpressed (NOV) (Brigstock et al., 2003)) are integral components of the ECM related to fibrosis and myofibroblast activation. Despite the designation as a growth factor, CCN2 is not a cytokine but an integral ECM protein that exerts its function through binding of cell integrins alone or recruitment of co-receptors (Jun and Lau, 2011). A number of reviews have summarized CCN2 functions in fibrosis (Chen et al., 2009; Gressner and Gressner, 2008; Shi-Wen et al., 2008). Expression of CCN2 (CTGF) is locally upregulated in a variety of fibrotic conditions and elevated in the serum of subjects with fibrosis. In addition, mutations in the CCN2 gene promoter are associated with systemic sclerosis in humans (Fonseca et al., 2007). Experimentally, blocking or deleting CCN2 efficiently reduces fibrosis, thus identifying CCN2 as a potential critical modulator of fibrosis. However, subsequent studies seem to suggest that activating functions of CCN2 occur either up- or down-stream of TGF-β1 signalling since simultaneous blocking of TGF-β1 abolished their myofibroblast activating effect (Hinz, 2007; Shi-Wen et al., 2008). TGFβ1 induces CCN2 expression in a variety of fibroblast culture and animal fibrosis models, nourishing the concept that CCN2 is a mere down-stream mediator of TGFβ1 in myofibroblast differentiation (Grotendorst and Duncan, 2005). However, different fibroblast culture models demonstrated expression of CCN2 in response to factors other than TGFβ1, such as endothelin-1 (Shi-wen et al., 2007).

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In many organs however, CCN2 seems to work synergistically with TGF-β1 in enhancing fibrosis but does not induce fibrosis and/or myofibroblast activation in the absence of TGFβ1 or injury (Brigstock, 2010; Wang et al., 2011).

1.6.9 Tenascin-C

Tenascin-C is a member of the tenascin family of ECM proteins (which also include tenascins-X, -R, and -W). Tenascin-C is classically regarded as a marker for the immature ECM in the earlier phases of tissue repair, promoting stromal cell population of provisional ECM by generating a migration-supporting adhesive environment and exerting chemokinetic effects (Yates et al., 2011). Indeed, tenascin-C plays a role in myofibroblast recruitment (Tamaoki et al., 2005).

Whereas tenascin-C is down-regulated in normally healing wounds, it persists in hypertrophic scar tissue where it seems to prevent cell apoptosis and prolongs the ECM synthesis and proliferative phase (Chiquet-Ehrismann and Chiquet, 2003; Yates et al., 2011). Tenascin-C null mice are protected against fibrosis in lung (Carey et al., 2010) and liver (El-Karef et al., 2007) with reduced amounts of α-SMA positive myofibroblasts. Less is known about the possible implication of other tenascin family members in myofibroblast biology and fibrosis. Tenascin-X knock-out mice exhibit reduced collagen amounts in skin dermis which shares phenotypic similarities with the human Ehlers-Danlos syndrome, including increased extensibility and reduced strength of the skin (Burch et al., 1997; Mao et al., 2002). Although cutaneous wounds of tenascin-X knock-out mice have reduced breaking strength, the contribution of myofibroblasts to the impaired biomechanical properties of the granulation tissue has not been tested yet (Egging et al., 2007).
1.6.10 Proteoglycans

Proteoglycans (including heparan sulphate proteoglycans, hyaluronan, syndecans, and small leucine-rich proteoglycans) are critical components of the wound healing response and are also implicated in tissue fibrosis. Experimental and mechanistic studies implicate these molecules in facilitating the assembly of matrices and the incorporation of growth factors (such as LTBP-1/TGF-β1 complexes) into the ECM (Chen et al., 2007).

Hyaluronan has long been associated with conditions of fibrosis, and hyaluronic acid (HA) is clinically used as a serum biomarker for liver fibrosis (Gressner et al., 2007). In addition, HA is purported to regulate myofibroblast activation and persistence in a TGF-β1-dependent manner (Simpson et al., 2009; Webber et al., 2009). The mechanisms of this action are not entirely clear, although fibroblast binding to HA positions the TGF-β1 receptor close to the HA receptor CD44, which affects downstream TGFβ1 signalling (Ito et al., 2004). HA also stabilizes cell-ECM adhesions (Twarock et al., 2010), which are crucial for myofibroblast mechanosensing and activation (Hinz et al., 2003). Fibroblasts deficient of the HA receptor CD44 displayed impaired migration, stress fibre formation and production of active TGF-β1, processes that are all dependent on cell adhesion (Acharya et al., 2008). Consistently, conditional overexpression of HA synthase 2 in α-SMA-positive lung myofibroblasts produced an invasive phenotype that promoted fibrosis progression in bleomycin-treated mouse lungs (Li et al., 2011). The same study showed that conditional deletion of HA synthase 2 under control of the Col1α2 promoter or inhibition of CD44 inhibited the aggressive myofibroblast phenotype and reduced development of fibrosis. Supported by these findings, HA signalling emerges as a novel target for therapeutic anti-fibrotic interventions.
Syndecans are another class of heparan sulphate proteoglycans that have been shown to affect organ fibrosis (Bartlett et al., 2007; Frangogiannis, 2010; Kliment and Oury, 2010). Shedding of syndecan-1 (CD138) by matrix metalloproteinases (MMPs) and oxidative stress was shown to contribute to fibrosis development (Fitzgerald et al., 2000; Kliment et al., 2009) and syndecan-1 supports FN fibrillogenesis (Stepp et al., 2010).

The direct effects of syndecans or syndecan fragments on myofibroblast activation have not yet been tested. However, syndecan-2 is known to modulate TGF-β signalling and TGF-β receptor expression presumably by directly binding to TGF-β1 (Chen et al., 2004). Furthermore, syndecan-4 knock-out mice exhibit reduced myofibroblast activation after myocardial infarct (Matsui et al., 2011) and in an animal model of lung fibrosis (Jiang et al., 2010).

Small leucine-rich proteoglycans (SLRPs) comprise a group of proteoglycans with a small protein core and unique tandem leucine-rich repeats. Among the best studied SLRPs are decorin, biglycan, lumican, and fibromodulin (Kalamajski and Oldberg, 2010). SLRPs fulfill a variety of functions that have a direct impact on ECM and cell homeostasis in fibrocontractive diseases; they regulate cell survival and collagen organization and they bind to growth factors, in particular TGF-β1 (Hildebrand et al., 1994; Iozzo and Schaefer, 2010). SLRPs are often up-regulated in different fibrotic conditions (Kalamajski and Oldberg, 2010) which contradicts the general observation that they act as negative regulators of myofibroblast activation. By contrast, SLRP are downregulated in dermal scarring correlating with fibrotic contractures (Honardoust et al., 2011).
This discrepancy may be explained by SLRP performing different functions in different phases of ECM remodelling during repair and fibrosis. For example, decorin potentially regulates myofibroblast activation by virtue of binding to active TGF-β1 (Yamaguchi et al., 1990).

Similarly, biglycan has anti-fibrogenic properties similar to decorin. Biglycan deficient cultured cardiac fibroblasts showed enhanced myofibroblast activation and contractile function due to increased TGF-β1 signalling (Melchior-Becker et al., 2011). Much less is known on the role of lumican in fibrocontractive diseases and regulating the myofibroblast phenotype, although it is upregulated during myofibroblast activation of corneal fibroblasts (Funderburgh et al., 1987).

1.6.11 Post-translational modification of the myofibroblast ECM

In addition to the composition of the ECM, mechanobiological properties also strongly dictate myofibroblast activation and function. Being contractile cells, myofibroblasts sense and modulate stiffness within the ECM through focal adhesions via integrin binding. Moreover, recent data suggest that mechanical stiffness alone, independent of TGF-β signalling, can induce myofibroblast activation in the setting of fibrosis (Liu et al., 2010). Thus, stiffness of the ECM is also a critical modulator of wound healing and fibrosis. Crosslinking of ECM proteins is the major determinant of tissue stiffening. Despite the low turnover rate of collagens in structural tissues such as skin and cartilage, crosslinking of ECM proteins (particularly collagens) is a potentially important area of exploitation for therapeutic purposes in fibrotic disorders.
Crosslinking ECM proteins may result in conformational changes that render epitopes ‘hidden’ from protease activity, thereby preventing digestion and remodelling of ECM. Thus, targeting enzymes and other proteins (discussed below) may provide a means by which fibrotic processes may be effectively halted or perhaps even reversed.

Transglutaminases (TGs) belong to a large family of proteins encoded by structural and functionally related genes (Grenard et al., 2001; Lorand and Graham, 2003). The major function of TGs is to catalyze the Ca\(^{2+}\)-dependent formation of inter-protein isopeptide bridges between γ-carboxyamide glutamine residues and ε-amino groups in the protein-bound lysine residues (Aeschlimann and Paulsson, 1994; Esposito and Caputo, 2005). TG2 is the most widely and ubiquitously expressed TG family member (Gundemir et al., 2012; Nurminskaya and Belkin, 2012). The ECM substrate spectrum of TG2 is large and comprises FN, vitronectin, collagens type I/II/V/VII/XI, laminin, fibrillin, and LTBP-1 to name only the most prominent (Esposito and Caputo, 2005; Wang and Griffin, 2012). Extensive cross-linking of collagen by TG2 produces collagen fibres that are resistant to degradation, and that support myofibroblast-mediated fibrosis (Huang et al., 2009b). In addition to the mechanical consequences of TG cross-linked ECM, the interaction of TG with fibrillins and LTBP-1 modulates deposition and activation of TGF-β1. Moreover, TGs are directly involved in the proteolytic activation of TGF-β1 from the large latent complex (Crawford et al., 1998), thereby potentially inducing myofibroblast differentiation. Other important enzymes that promote ECM protein cross-linking in normal and pathological tissue repair belong to the lysyl oxidase (LOX) and lysyl oxidase-like (LOXL) families.
LOX is a copper-dependent amine oxidase that forms reactive aldehyde groups from peptidyl lysines in its substrates by oxidative deamidation; these reactive groups spontaneously form covalent cross-links (Kagan, 1994; Smith-Mungo and Kagan, 1998). The covalent cross-linking of fibrillar collagen by LOX is of particular importance in fibrotic disease progression (Kagan, 2000). LOX is upregulated in conditions of tissue repair and fibrosis (Lopez et al., 2010) and induced by TGF-β1 in fibroblast cultures (Goto et al., 2005; Roy et al., 1996; Voloshenyuk et al., 2011). Furthermore, LOX plays a key role in promoting fibroblast-to myofibroblast activation in skin, heart, liver, kidney and lung fibrosis (Chanoki et al., 1995; Counts et al., 1981; Di Donato et al., 1997; Kagan, 1994). The conversion of fibroblast-secreted collagen into insoluble fibres by LOX contributes to the accumulation of stiff ECM and thereby contributes to the progression/persistence of fibrosis (Lopez et al., 2010; Lopez et al., 2012; Noblesse et al., 2004). In addition to LOX, LOXL2 has been recently identified to form fibrosis-specific and stable collagen cross-links (Barker et al., 2011). LOXL2 oxidatively deaminates the ε-amine group of specific lysine residues of collagen and elastin (Vadasz et al., 2005). Collagen crosslinking also occurs without enzymatic support by glycation; although this process is comparably slow, it is physiologically relevant given the low turnover time of collagen with a half-life of 15 years in skin and one order of magnitude longer in cartilage (Sivan et al., 2008; Verzijl et al., 2000). A variety of fibrotic and pre-fibrotic conditions such as diabetes are characterized by pathological levels of advanced glycation end-products (AGE) and tissue stiffening due to glycation (van Heerebeek et al., 2008). AGE are pro-fibrotic in that they promote production of type I and type III collagens (Singh and Mascarenhas, 2008; Tang et al., 2007), increase fibroblast proliferation (Tokudome et al., 2004), induce TGF-β1-
dependent and –independent fibrotic changes (Chung et al., 2010; Martin and Leibovich, 2005), and induce collagen glycation (Schnider and Kohn, 1980).

1.7 TGF-β1 biology

The ECM provides structural support for cells and has a variety of different functions depending on the tissue which is reflected in the ECM’s composition and structure. During wound healing, myofibroblasts and inflammatory cells secrete various cytokines and growth factors that are stored in the ECM (Werner and Grose, 2003). TGF-β1 is a paradigm cytokine that is stored in a latent form in the ECM. The TGF-β family comprises multipotent cytokines modulating cell growth, inflammation, ECM synthesis and apoptosis (Taipale et al., 1998). In mammals, these functions are mediated by the three widely expressed isoforms TGF-β1, -β2 and -β3 (Annes et al., 2003). TGF-β1 stimulates the synthesis of ECM proteins, regulates tumor development and is upregulated in fibrotic conditions, in scar formation, and wound healing (Raghunath et al., 1998; Yang, 2010). TGF-β1 is the most potent inducer of myofibroblast differentiation (Desmouliere et al., 1993). TGF-β1 is secreted as a large latent complex (LLC) by a great variety of different cell types, including fibroblasts and myofibroblasts. The LLC consists of TGF-β1, which remains non-covalently associated with its pro-peptide, the latency associated protein (LAP). LAP covalently binds to the latent TGF-β1 binding protein (LTBP) which in turn mediates ECM incorporation (Figure 5) (Wipff and Hinz, 2008). In addition to the binding of integrins to LAP, RGD sequences have been identified in the LTBP moiety of the LLC (Sheppard, 2005; Wipff and Hinz, 2008).
Figure 5: TGF-β1 is secreted in a large latent complex.

TGF β1 is secreted in a large latent complex (LLC). The LLC consists of TGF-β1, associated with LAP-β1 and the LTBP-1. Together, LAP-β1 and TGF-β1 form the small latent complex (SLC) which is intracellularly formed as a homodimer linked by disulfide bonds. LAP β1 contains four mannose-6-phosphate moieties that are required to associate with TGF-β1. LAP-β1 and LAP-β3 contain the amino acid sequence motif RGD serving as recognition site for various integrins. LTBP-1 contains three cysteine-rich domains, 14 Ca$^{2+}$-binding EGF-like domains, three non Ca$^{2+}$-binding EGF-like domains, and one hybrid domain. The second cysteine-rich domain binds the SLC via disulfide bonds whereas the third cysteine-rich domain at the N-terminus of LTBP-1 links the protein complex to the ECM via transglutamination. The flexible hinge region, separating the central hybrid domain and the third cysteine-rich domain, is sensitive to proteolytic digestion (arrowhead). Another protease-sensitive site is present close to the C-terminus of LTBP-1 (arrowhead). Adapted from Wipff and Hinz, 2008 (Wipff and Hinz, 2008).

Blocking specific integrins has been proposed as a powerful strategy to control TGF-β1 availability in physiology and disease rather than targeting the active pleiotrophic cytokine (Hinz, 2009; Varga and Pasche, 2009).

1.8 Integrins in TGF-β1 activation

We have previously demonstrated that lung myofibroblast contraction can directly activate TGF-β1 from self-generated latent stores in the ECM (Taipale et al., 1998).
This activation mechanism requires binding of latent TGF-β1 to a stiff ECM and transmission of cell forces to latent TGF-β1 via integrins (Taipale et al., 1998). During pulmonary fibrosis, the epithelial integrin αvβ6 was identified to activate TGF-β1 by binding to LAP (Munger et al., 1999). In fibroblasts that do not express αvβ6, only integrins αvβ5, αvβ3 and α8β1 are possible candidates for direct TGF-β1 activation since only these fibroblastic integrins bind to RGD. It was shown that the integrins αvβ3 and αvβ5 contribute to autocrine TGF-β1 signaling in scleroderma fibroblasts (Asano et al., 2006; Asano et al., 2005). The integrin αvβ5 was found to be mainly involved in TGF-β1 activation by cultured lung myofibroblast (Wipff et al., 2007). Our laboratory showed that the integrins αvβ5 and αvβ3 both control human cardiac myofibroblast differentiation by activating latent TGF-β1 (Sarrazy et al., 2014). Furthermore, integrin α8β1 was found to bind to TGF-β1 storage complex (Lu et al., 2002). In addition to the state of myofibroblast contractile activity and the nature of the integrin anchors with LLC, the stiffness of the ECM dictates the amount of TGF-β1 that can be activated (Wipff et al., 2007). In fact, the deletion of the αv subunit specifically in myofibroblasts derived from hepatic stellate cells protected mice from carbon tetrachloride-induced hepatic fibrosis (Henderson et al., 2013). Myofibroblast are not able to liberate active TGF-β1 from a compliant ECM that appears to absorb cell-generated deformation forces. Under normal conditions, fibroblast are shielded by the ECM architecture but become exposed to considerable stress when the protective ECM structure is lost after injury (Tomasek et al., 2002). During wound healing, myofibroblast remodelling activities stiffen the ECM to an extent that is comparable towards the end of wound healing with hypertrophic scar and fibrotic tissues (Goffin et al., 2006).
Chapter 2
A Cell-Free System to Activate TGF-β1 from the Extracellular Matrix

The contents of this Chapter have been published in:


Current Biology, 2011 December 20; 21(24):2046-5

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My contribution to this work as co-second author has been to develop and use an assay that enables proof of concept experiments in releasing TGF-β1 mechanically without cells. The assay mimics the release of TGF-β1 from the latency complex by pulling via integrins on a global level compared to the employed atomic force microscope studies on the single molecule level. Only these parts are reproduced in my thesis. The experiments I have performed in this work were essential for my thesis to demonstrate that TGF-β1 release via integrins is based on a mechanical principle and to investigate how ECM organization changes and different ECM strain conditions and the influence on TGF-β1 release (Chapter 3).
2.1 Abstract

TGF-β1 controls many pathophysiological processes including tissue homeostasis, fibrosis, and cancer progression. Together with its LAP, TGF-β1 binds to the LTBP-1, which is part of the ECM. Transmission of cell force via integrins is one major mechanism to activate latent TGF-β1 from ECM stores. Latent TGF-β1 mechanical activation is more efficient with higher cell forces and ECM stiffening. However, little is known about the molecular events involved in this mechanical activation. By using αvβ6-integrin coated microbeads, we were able to activate TGF-β1 from a pre-produced, de-cellularized ECM via application of magnetic forces. 30% of total TGF-β1 was activated when compared to force application with BSA coated microbeads. Our results confirm a purely mechanical mechanism of TGF-β1 activation. Integrin mediated force transmission proves to be sufficient to open the complex in the absence of cells. Interfering with mechanical activation of latent TGF-β1 by reducing integrin affinity, cell contractility, and binding of latent TGF-β1 to the ECM provides new possibilities to therapeutically modulate TGF-β1 actions.
2.2 Introduction

TGF-β1 is a pleiotropic cytokine that controls cell growth, inflammation, tissue homeostasis, and immune suppression in a variety of normal and pathologic adult tissues (Ikushima and Miyazono, 2010; Li et al., 2006; Moustakas and Heldin, 2009; Yang, 2010). TGF-β1 is also an important therapeutic target because of its involvement in the pathogenesis of many disorders, including organ fibrosis and tumorigenesis. The unsuccessful outcomes of blocking the already active TGF-β1 in animal experiments and clinical tests in combination with the risk of uncontrollable side effects has shifted research toward preventing latent TGF-β1 activation in a cell type- and activation mechanism specific manner (Howell and McAnulty, 2006; Meier and Nanney, 2006; Nishimura, 2009a; ten Dijke and Arthur, 2007; Varga and Pasche, 2009; Wakefield and Stuelten, 2007; Worthington et al., 2011). TGF-β1 is synthesized as part of a proprotein that is intracellularly cleaved to produce the SLC. Mature SLC consists of the TGF-β1 dimer, non-covalently linked to the dimeric LAP (Figure 1). Most cell types secrete SLC together with LTBP-1, constituting the LLC (Annes et al., 2003; Hyytiainen et al., 2004). LTBP-1 targets latent TGF-β1 to the extracellular matrix (ECM) by interacting with different proteins including fibronectin and fibrillin (Hynes, 2009; Ramirez and Rifkin, 2009), generating deposits of latent TGF-β1 accessible force-mediated activation (Annes et al., 2004b; Jenkins, 2008; Wipff and Hinz, 2008). Integrins have emerged as central players in TGF-β1 activation (Nishimura, 2009a; Sheppard, 2005; Wipff and Hinz, 2008).
The expression of cell type-characteristic integrins provides means to interfere with TGF-β1 activation in a tissue- or cell-specific manner (Asano, 2010a; Goodwin and Jenkins, 2009; Nishimura, 2009a; Wipff et al., 2007; Zhou et al., 2010). Epithelial cells activate latent TGF-β1 via integrin αvβ6 (Aluwihare and Munger, 2008; Annes et al., 2004b), whereas fibroblasts, devoid of αvβ6 integrin, appear to promote latent TGF-β1 activation through integrins αvβ3, αvβ3 and αvβ5 (Asano, 2010a; Goodwin and Jenkins, 2009; Nishimura, 2009a; Wipff et al., 2007; Zhou et al., 2010). Integrins activate latent TGF-β1 by at least two different mechanisms (Sheppard, 2005; Wipff and Hinz, 2008). One depends on proteases, which seem to be guided to the LLC by associating with integrins (Jenkins, 2008; Nishimura, 2009a).

The other is independent of proteolysis and involves transmission of cell traction forces to the LAP moiety of LLC (Jenkins, 2008; Sheppard, 2005; Wipff and Hinz, 2008). We and others have proposed that ECM-bound LTBP-1 provides mechanical resistance against cell-mediated contraction to induce a conformational change in LAP that leads to liberation of active TGF-β1 (Annes et al., 2004b; Shi et al., 2011; Wipff et al., 2007). This mechanical mode of action seems to play a major role in stiff fibrotic scars (Hinz, 2010b; Wipff and Hinz, 2008; Wipff et al., 2007).
2.3 Materials and Methods

2.3.1 Plasmid constructs, cell culture and transfection

To allow live detection, we produced fluorescent fusion proteins of all human LLC; EYFP-LAP-β1 (EYFP-LAP) was commercially obtained (IOH4479-pdEYFP-C1amp, imaGenes GmbH, Germany). Recombinant proteins were expressed in Chinese hamster ovary (CHO K1) cells after transient transfection via JetPei (PolyPlus-transfection SA, France) according to manufacturer’s instructions. CHO cells, stably transfected with LTBP-1S (CHO-LTBP-1), were a kind gift from D. Rifkin. We used 10 mg/ml recombinant SLC/LAP to coat mica surfaces (R&D Systems, MN). Lab reagents were obtained from Sigma (Switzerland).

2.3.2 ECM Preparation, Immunofluorescence Staining, and Microscopy

ECM containing different LLC components was produced by plating LTBP-EGFP transfected CHO cells for 7 days on glass coverslips or on 35mm“m-dishes” with grid (IBIDI GmbH, Germany) in serum-free conditions. Pure ECM was obtained by removing cells with ice-cold desoxycholate (DOC) buffer (0.5% DOC, 1% NP-40, 150 mM NaCl in 10 mM Tris-HCl [pH 8.0]) for 10 min, followed by washing with PBS. Only protein layer intimate to the culture surface were preserved after DOC extraction of CHO cells. We have preferred this approach over using purified LTBP-1 and LLC to preserve proper folding and conformation of protein in complexes and to avoid loss of TGF-β1 from the LLC during purification.
Extracellular LLC components on whole-cell preparations were immunostained by incubating living cells with the corresponding primary antibodies (2h, 37°C), followed by fixation and incubation with secondary antibodies omitting permeabilization. LLC components from fresh DOC-extracted samples were stained similarly. Fluorescence imaging was performed with a SP2 Leica AOBS inverted confocal microscope (Leica Microsystems GmbH, Germany) and deconvoluted with HuygensPro (SVI, The Netherlands).

2.3.3 TGF-β1 activity measurements

To measure active TGF-β1, transformed mink lung epithelial cells (TMLC) reporter cells (Abe et al., 1994) were used. TMLC cells contain the plasminogen activator inhibitor-1 (PAI-1) promoter fused to the luciferase reporter gene. The amount of expressed luciferase molecules inside the cells is equivalent to the number of TGF-β1 molecules binding to the TGF-β1 receptors. To measure total TGF-β1, all experimental samples were run with a control condition (separate dish), which was heated to 80°C for 10 min to release all TGF-β1 from the latent complex (Wipff et al., 2007). In addition, each experiment included a TGF-β1 standard curve to determine TMLC efficacy. After TMLC were incubated with experimental condition samples and total TGF-β1 samples for 16h in the absence of FBS, the TMLC were lysed and luciferase activity was quantified with a luciferin substrate (Promega, WI) in a CentroLB Luminometer (Berthold Technologies, Germany). The amount of active TGF-β1 was calculated as percentage of total TGF-β1 in the experimental control condition. TGF-β1 levels are expressed as mean±SD of at least three independent experiments performed in duplicates.
2.3.4 Force application via ferromagnetic microbeads

To directly demonstrate force-mediated release of active TGF-β1 from LLC ECM, we used ferric oxide microparticles (Sigma) and a permanent ceramic magnet. Beads were coated with 10 mg of LAP antibodies (R&D Systems), recombinant human integrin αvβ6 (R&D Systems), or BSA (BioShop Canada Inc., ON). Beads were then washed in PBS and resuspended in fresh serum-free TMLC medium. DOC-extracted ECM of LLC-expressing cells was incubated with coated beads (30 min) before multidirectional force was applied with the magnet. TGF-β1 released from LLC was measured with TMLC reporter cells.

2.4 Results

2.4.1 Unfolding of recombinant SLC and LAP

In order to test whether TGF-β1 binding to LAP changes the unfolding mechanics of the SLC, we first compared the unfolding profiles of recombinant SLC (TGF-β1-loaded) and LAP (empty) adsorbed onto mica substrates. SLC and LAP were then pulled with AFM probes covalently coated with anti-LAP monoclonal antibodies and the increase in protein contour length (ΔLc) was determined (Figure 6C). Three different unfolding populations for SLC were found, 7.1±1.6 nm, 11.1±2.8 nm, and 17.5±7.9 nm. Similarly, three length change population were found for LAP at 12.0±2.6 nm, 19.0±4.5 nm, and 26.9±9.5 nm.
Force correlation due to data transformation into bivariate plots revealed clear differences between SLC and LAP unfolding characteristics. SLC unfolding presents one main event population at 8.1 nm/44.9 pN, whereas LAP unfolding showed three populations at 11.0 nm/46.5 pN, 13.6 nm/74.1 pN, and 12.1 nm/115.5 pN.

2.4.2 Analysis of LAP Structural Domains

These different unfolding characteristics suggest that LAP attains a stable new conformation after TGF-β1 release. We subsequently analyzed whether the ΔLc measured in recombinant LAP and SLC corresponded to the lengths of important structural domains of the LAP moiety. Recently published 3D structure data indicate that the LAP moiety is folded in two principal regions, the straitjacket (Leu30-Pro74) and the arm (Glu75-Arg278) (Shi et al., 2011) (Figure 6A).
See legend next page
Figure 6: Single-molecule mechanical stretch opens the latent TGF-β1 complex to release TGFβ1.

(A) Crystal structure of the small latent complex SLC. TGF-β1 is trapped by LAP in a flexible “straitjacket” configuration (red ellipse) (Shi et al., 2011). (B) Domains that are prone to unfolding within the straitjacket are the latency lasso and the α-helix. (C) Atomic force microscope cantilever tips were used to stretch the LLC. (D) Typical force-extension profiles of LLC pulling. Every sawtooth indicates unfolding of one protein domain. (E) All force-extension curves were analyzed for unfolding lengths (ΔLc) that correspond to the length of the whole 45 amino acid (aa) straitjacket, the 17 aa latency lasso and the 25 aa α-helix domain. Values were plotted in a bivariate ΔLc-force contour map with red indicating high occurrence of unfolding events. (F) In a nutshell, the experiments demonstrate that forces of >40 pN unfold the straitjacket and thereby liberate active TGF-β1 (Buscemi et al., 2011b).

Of those, only the α1 helix (Leu30-Arg58) and the latency lasso (Leu59-Pro74) domains within the straitjacket are expected to unfold under stretch (Figure 6B). Based on these premises, we analyzed whether the ΔLc experimentally measured in recombinant LAP and SLC corresponded to the lengths of any of these structural domains. A pulling force of 47.8 pN was required to unfold the 6.8 nm latency lasso domain and a force of 80.8 pN to unfold the 11.2 nm α1 helix domain of SLC.

2.4.3 Mechanical Activation of TGF-β1 from the ECM

Cell-mediated mechanical activation of latent TGF-β1 was shown to require immobilization of the SLC in the ECM via covalent binding to LTBP-1 (Annes et al., 2004b; Wipff et al., 2007), which potentially alters the force needed to unfold SLC. We first established the profile of force-induced unfolding of LTBP-1 alone to later discriminate unfolding events in the SLC/LAP moiety of the LLC from LTBP-1 unfolding events (Figure 6D). Therefore, the AFM probe was specifically targeted to mRFP-labelled LTBP-1 after cells were removed with DOC. Bivariate plots of ΔLc unfolding force pairs per every single event displayed three distinct peaks at 16.7 nm/179.4 pN, 22.6 nm/68.2 pN, and 34.5 nm/39.8 pN.
Thus, unfolding of the LTBP-1 resulted in a combination of short stretch-resistant domains and long flexible domains. Importantly, all these unfolding events were distinct from those observed in the recombinant SLC/LAP proteins. Summarizing the LLC (LTBP-1/SLC/LAP) data in $\Delta$Lc-force pair plots revealed two dominant populations at 14.8 nm/61.2 pN and 18.8 nm/43.7 pN and filtering for LAP/SLC domain unfolding events (Figure 6E) delivered two event populations corresponding to the a1 helix domain (11.4 nm/51.2 pN) and to the whole straitjacket (18.3 nm/38.6 pN) (Figure 6B). It has been predicted from the SLC crystal structure that straitjacket opening will necessarily lead to TGF-β1 release. Our data together with the crystal structure of SLC indicate full opening of the LAP straitjacket upon LLC pulling, supporting an “all-or-nothing” model of TGF-β1 release upon activation (Figure 6F).

2.4.4 TGF-β1 activation in a cell-free model

Due to the fact that one single TGF-β1 molecule is below the detection sensitivity of TMLC reporter cells, I developed a different pulling assay. Ferromagnetic beads were coated with LAP antibody or with recombinant integrin αvβ6, the most potent LAP-binding and TGF-β1 activating integrin (Annes et al., 2004b; Munger et al., 1999). BSA-coated microbeads were used as controls. Microbeads were adsorbed onto DOC-extracted LLC-rich ECM. All controls with no force application exhibited low levels of active TGF-β1 in the medium, reaching 15–20% of total TGF-β1 available in the ECM. Applying magnetic force to BSA coated beads led to the release of active TGF-β1 of ~25% of total TGF-β1 (Figure 7), suggesting that non-specific shear forces are able to activate TGF-β1 (Ahamed et al., 2008).
Figure 7: Integrin-Mediated Mechanical TGF-β1 Activation.

(A) Ferromagnetic 1 μm diameter microbeads were coated with anti-LAP antibodies, recombinant integrin αvβ6 (inset scheme, not to scale), or BSA (control). Microbeads adsorbed to DOC-extracted LLC-rich ECM were subjected to a magnetic field (force) or left without magnet (no force). TGF-β1 release was measured after 30 min via TMLC reporter cells and related to total TGF-β1 available from the ECM after heat activation. Shown are means±SD (*p % 0.05, **p % 0.01, Student’s t test).

In contrast, pulling anti-LAP- and αvβ6 integrin-coated beads released 1.7- to 2.2-fold higher quantities of active TGF-β1 (~40% and 50% of total TGF-β1, respectively) compared with BSA-coated magnet-pulled beads (Figure 7).
2.5 Discussion Chapter 2

We have previously established, that transmission of cell contractile forces to LAP via integrins activates latent TGF-β1 reservoirs from noncompliant ECM (Wipff et al., 2007). Furthermore, our data together with the crystal structure of SLC indicate full opening of the LAP straitjacket upon LLC pulling, supporting an “all-or-nothing” model of TGF-β1 release upon activation (Shi et al., 2011). The question remains how mechanical TGF-β1 activation can be regulated in such a model.

One possibility could be interference with the opening of the TGF-β1 complex. In this scenario, TGF-β1 release from the LLC need to overcome a pulling force threshold, which regulation probably occurs at the level of force build-up. This control can imply modulation of (1) intracellular force development by actin/myosin interaction, (2) integrin binding affinity and strength by feedback force or chemical signals, and (3) ECM stiffness and resistance to integrin pulling.

All these premises are in agreement with our previous observation that TGF-β1 activation is modulated by the level of cell-generated tension and that ECM stiffness has to exceed a threshold to result in mechanical TGF-β1 activation (Wipff et al., 2007). A compliant ECM does not oppose cell-derived contractile forces and LAP unfolding will be impaired, regardless of the cell contraction strength. A stiff ECM, however, provides the necessary resistance to promote a full conformational change of the LAP and TGF-β1 release by cell contraction. Hence, one integrin would be sufficient to transmit the force required to unfold the latent TGF-β1 structure.
Force values, obtained with single-molecule atomic force microscopy, are different from values obtained from the microbead pulling experiments, which typically deliver at least 10- to 100-fold lower integrin forces (Moore et al., 2010; Weisel et al., 2003). This discrepancy partly originates from the fact that microbead experiments average force values over the number of possible integrin-ligand interactions on the bead surface. However, not all integrins on the bead surface will be bound, leading to a systematic underestimation of single integrin force. Importantly, magnetic microbeads can exert the forces that are needed to directly activate and release TGF-β1 from a LLC ECM in our experiments, demonstrating for the first time mechanical TGF-β1 activation in a cell-free system.

In summary, we have proven that the mechanical stress that cells transmit onto the integrin receptors is transmitted to the latent TGF-β1 complex leading to the liberation of active TGF-β1. However, the impact of integrin affinity state in this process remains elusive and provides the basis to further research. Nevertheless, we assume that mechanical activation of latent TGF-β1 is still restricted to integrins with high binding affinity to LAP, to cells that can transmit considerable force to the LAP, and to a sufficiently rigid ECM. Hence, pharmacological interference with either factor will affect TGF-β1 activation. Importantly, therapeutic strategies to block harmful TGF-β1 activity in fibrosis or cancer progression could already be effective when integrin binding and cell contraction are below the TGF-β1 activation threshold.
Chapter 3
Pre-Stress in the Extracellular Matrix Sensitizes Latent TGF-β1 for Activation

The contents of this chapter have been published in:

“Pre-Stress in the Extracellular Matrix Sensitizes Latent TGF-β1 for Activation”

Journal of Cell Biology, 2014 (in press)

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My newly developed, cell-free TGF-β1 activation assay enabled me to test the hypothesis that mechanical stress in the ECM impacts TGF-β1 release. Chapter 3 comprises the main body of this thesis, addresses objective 2 and shows how myofibroblasts remodel the ECM for enhanced TGF-β1 activation. I analysed \textit{in vivo} samples from rat wounds and perform \textit{in vitro} experiments with the aim to find changes in the ECM composition and structure. Labeling LTBP-1 with GFP helped me to visualize LTBP-1 fibril formation over time and purification of LTBP-1 in combination with micro-contact printing LTBP-1 identified binding integrins. I aimed to prove that changes in the ECM have an impact on TGF-β1 release and showed that with \textit{pre-stressed} vs. \textit{non-stressed} ECM. I established a link between ECM organization, LTBP-1 fibril strain and the amount of active TGF-β1 that can be released. This mechanism provides a crucial checkpoint for the bioavailability of TGF-β1.
3.1 Abstract

Integrin-mediated force application induces a conformational change in latent TGF-β1 that leads to the release of the active form of the growth factor from the extracellular matrix (ECM). Mechanical activation of TGF-β1 is currently understood as an acute process that depends on the contractile force of cells.

However, we show that ECM remodeling, preceding the activation step, mechanically primes latent TGF-β1 akin to loading a mechanical spring. Cell-based assays and unique strain devices were used to produce cell-derived ECM of controlled organization and pre-strain. Mechanically conditioned ECM served as substrate to measure the efficacy of TGF-β1 activation following cell contraction or direct force application using magnetic microbeads. The release of active TGF-β1 was always higher from pre-strained ECM as compared to unorganized and/or relaxed ECM.

The finding that ECM pre-strain regulates the bio-availability of TGF-β1 is important to understand the context of diseases that involve excessive ECM remodeling, such as fibrosis or cancer.
3.2 Introduction

Myofibroblasts contribute to normal tissue repair by replacing and contracting the provisional ECM that fills tissue defects after injury (Hinz et al., 2012). When ECM remodelling activities of myofibroblasts are deregulated, repair proceeds into adverse and pathological fibrosis affecting all organs, including skin, heart, lung, liver, and kidney (Hinz et al., 2012; Wynn and Ramalingam, 2012). TGF-β1 is the most potent pro-fibrotic cytokine known and the main growth factor inducing myofibroblast differentiation from a variety of different precursor cells (Hinz et al., 2007). Fibroblasts secrete TGF-β1 non-covalently associated with its LAP. This small latent complex covalently binds to the LTBP-1, an integral component of the ECM that stores and presents latent TGF-β1 for subsequent activation (Jenkins, 2008; Robertson and Rifkin, 2013; Worthington et al., 2011; Zilberberg et al., 2012a). Binding of LAP to the ECM through the LTBP-1 is the structural pre-condition for mechanical activation by integrins (Annes et al., 2004a; Shi et al., 2011; Wipff et al., 2007). The LTBP-1 binding site of LAP is directly opposite to the RGD site in LAP for integrin attachment; integrin-mediated force transmission induces a conformational change in LAP that liberates active TGF-β1 (Buscemi et al., 2011b; Shi et al., 2011). All αv-integrins bind to RGD in LAP (Henderson and Sheppard, 2013; Hinz, 2013; Jenkins, 2008; Nishimura, 2009b; Wipff and Hinz, 2008). Integrins αvβ3, αvβ5, αvβ6, and possibly αvβ1 activate latent TGF-β1 by transmitting cell contractile forces (Giacomini et al., 2012; Henderson et al., 2013; Wipff et al., 2007).
We have previously demonstrated that the acute contractile state, i.e., the force exerted by fibroblastic cells, determines the quantity of TGF-β1 that is activated from the ECM (Buscemi et al., 2011b; Wipff et al., 2007). Here, we propose that the changes in ECM organization produced by fibroblastic cells over days, weeks, and months in fibrotic lesions will augment the bio-availability of TGF-β1. We show that myofibroblasts mechanically prime TGF-β1 for activation by actively organizing the latent complex in the ECM during and after secretion, analogous to the loading of a mechanical spring. High levels of experimentally controlled ECM organization and mechanical load always resulted in high levels of TGF-β1 activated by acutely contracting myofibroblasts.

Our results suggest that the excessive remodelling activity of fibroblastic cells in the early stages of tissue repair will set the stage for the development of fibrosis by adjusting the mechanical trigger point for latent TGF-β1 activation.

3.3 Materials and Methods

3.3.1 Ethics statement

Human material was provided by Dr. Benjamin Alman and the use was approved by the Institutional Review Board of the Hospital for Sick Children. Written informed consent was obtained from patients for the use of the material.

3.3.2 Animal experiments

On a total of 20 female Wistar rats (200-220 g), full thickness 20x20 mm wounds were produced in the middle of the dorsum.
Wounds were allowed to heal either spontaneously (control) or were subject to mechanical stress by fixing the edges of the wound tissue on a plastic frame that prevents wound closure and retains the size of the open wound over time (stressed). Authorization of the local animal ethic committees was obtained as previously described (Hinz et al., 2001b). Rats were sacrificed by CO₂ anesthesia and wound granulation tissue was harvested after 3d, 6d and 9d post-wounding. For immunofluorescence staining of rat tissues, 5 µm sections were fixed with 100% acetone for 15 min at -20°C and then dried for 30 min at RT. After adjusting to room temperature, sections were rinsed in PBS, blocked with 1% BSA for 30 min, and stained with primary antibodies ED-A FN (1:200, Santa Cruz Biotech, Cat#sc-59826) and LTBP-1 (1:100, R&D Systems, Cat#MAB388 or 1:250, Ab39, gift from Carl-Hendrik Heldin, Uppsala University, Sweden), followed by 30 min secondary fluorescently labelled antibodies.

3.3.3 Cell culture and reagents

Fibroblasts were explanted from human dermal tissue derived human tissue sections. In brief, tissues were cut into 1 mm³ cubes, attached to tissue culture plates, and immersed in standard Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies), supplemented with 10% fetal bovine serum (Sigma-Aldrich), and penicillin/streptomycin (Life Technologies). Cells were allowed to migrate out of tissues for 10d before the first passage. To promote hDF-to-hDMf differentiation, TGF-β1 (2 ng/ml, R&D Systems, Cat#100-B-001) was added for 6 d. The Flp-In™ 293 cell line (gift from P. Jurdic, Université de Lyon, France) was maintained in standard medium supplemented with Zeocin™ (1:1000, Life Technologies, Cat#R250-05).
Flp-In™ 293 cells from a single cell clone were transfected with pSecTag-LTBP-1-EGFP and a stable HEK293-LTBP-1-EGFP (HEK-LTBP-1) cell line was derived by fluorescence activated cell sorting and limited dilution. HEK293-LTBP-1 cells were maintained under hygromycin B (1:50, Life Technologies, Cat#10687-010) selection. Human dermal fibroblasts and myofibroblasts, wild-type MEF (ATCC, CRL-2645™), FAK−/− (ATCC, CRL-2644™), filamin A knock-down, created by stable transfection of NIH3T3 cells with shRNA against filamin A were gift (Kiema et al., 2006; Shifrin et al., 2009) and integrin β1−/− knock out cells (GD25) (Fassler et al., 1995) were gifts from C. McCulloch, University of Toronto. For integrin β1−/− knock out cells, the second exon of the β1integrin gene in embryonic stem (ES) cells was disrupted with a gene trap vector with a β-galactosidase-neomycin fusion DNA. ES cells were immortalizing with recombinant retroviruses that transduced the SV-40 large T. A single clone was established that was mutated in both alleles. The homozygous mutant clone did not produce integrin β1 mRNA or protein (Fassler et al., 1995). FN−/− MEF were a gift from Mercedes Costell (Universitat de València, Spain). In brief, ES cells were isolated form E14.5 FN floxed embryos, immortalized via retroviral transduction of SV large T antigen, cloned and treated with adenoviral Cre to delete the floxed FN alleles. The fibronectin targeting vector was spanning the region from the promotor to the second intron.

For the floxed fibronectin allele, loxP sites were confirmed within the 5′-untranslated region and within the first intron. Cre-mediated recombination at these two loxP sites removed the start codon, signal sequence and the exon/intron border of exon 1 to generate the null allele. (Sakai et al., 2001).
Wild-type and fibrillin-1 C1039G/+ mutant mouse dermal myofibroblasts (gift from H. Dietz, Johns Hopkins University School of Medicine, Baltimore, MD) (Judge et al., 2004) were maintained in standard culture media.

3.3.4 Plasmids and purification of LTBP-1-EGFP

The pSecTag-LTBP-1-EGFP plasmid was created by subcloning the previously published LTBP-1-EGFP sequence in frame of pSecTag/FRT/V5-His-TOPO® vector (Life Technologies) (Buscemi et al., 2011b). Correct integration was confirmed by sequencing the insert both ways at TCAG (SickKids Hospital, Toronto, Canada). LTBP-1 was purified from serum-free conditioned media of HEK-LTBP-1. In brief, conditioned medium was collected and dialyzed against DBPS (Life Technologies) before it was run over an ion metal affinity chromatography column (IMAC) with HIS-Select® Nickel Affinity Gel (Sigma Aldrich, Cat#P6611). Columns were washed with wash buffer containing 0, 10, or 15 mM imidazole. Fractions containing LTBP-1 were eluted with 250 mM imidazole (Figure 12).

3.3.5 Immunofluorescence

Preceding immunofluorescence staining of cultured cells, samples were simultaneously fixed and permeabilized in 3% paraformaldehyde. In vitro samples were fixed with 3% paraformaldehyde for 10 min, washed with PBS, and then permeabilized with 0.2% Triton X-100 for 5 min. Primary antibodies used in this study were: α-SMA (mouse IgG2a, clone SM1, a kind gift of Giulio Gabbiani, University of Geneva, Switzerland), vinculin (rabbit, 1:400, Abcam, Cat#ab11194), FN (rabbit, 1:400, Sigma-Aldrich, Cat#F3648), ED-A FN (mouse IgG1,1:200, Santa Cruz Biotech, Cat#sc-59826),
LTBP-1 (mouse IgG1, 1:100, R&D Systems, Cat#MAB388 or rabbit, 1:250, Ab39), fibrillin-1 (rb, 1:500, raised against recombinant, soluble N-terminal half of fibrillin-1 fragment (rFBN1-N), gift from Dr. Dieter P. Reinhardt, McGill University, Canada) (Sabatier et al., 2009), integrin β1 (mouse IgG1, 1:50, Abcam, Cat#ab30394), integrin β3 (mouse IgG1, 1:50, Millipore, Cat#MAB1974) and integrin β5 (rabbit, 1:50, Abcam, ab15459). Recombinant LTBP-1 was stained with anti-GFP antibodies (rabbit, 1:200, Abcam, ab290 and mouse IgG1, 1:200, Abcam, ab291) to achieve higher detection sensitivity than anti-LTBP-1 antibodies. Secondary antibodies used were: goat anti-mouse IgG Alexa Fluor 568 (1:100, Life Technologies, Cat#A-11004), goat anti-mouse IgG1 FITC (1:100, Southern Biotechnology, Cat#1070-02), goat anti-mouse IgG2a TRITC (1:100, Southern Biotechnology, Cat#1080-03) and goat anti-rabbit-TRITC and -FITC (1:100, Sigma-Aldrich, Cat#F9887). To stain filamentous actin and nuclear DNA, phalloidin Alexa Fluor 488 and 568 (1:100, Life Technologies, Cat#A-12379 and A-22287) and 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (1:50, Sigma-Aldrich, Cat#D9542) were used, respectively.

3.3.6 Image acquisition, processing and quantitative analysis

Fluorescence microscopy images were acquired with an Axio Imager upright microscope equipped with an AxioCam HRm camera, Apotome 2 structured illumination and ZEN software (“Zeiss-1”, Zeiss, Oberkochem, Germany). Figure 8A images were taken with Plan-Apochromat objectives (Zeiss, 40X, NA 1.2, top two rows, and Zeiss, 63X, NA 1.4, Oil-DIC, RT) on Zeiss-1 using Apotome structured illumination to calculate confocal optical sections. Figure 8B (top) images were taken with a Fluar objective (Zeiss,
20X, NA 0.75, RT, air) on Zeiss-1. Figure 8B (bottom) images were taken with a Plan Fluar objective (Nikon, 40X oil immersion, NA 1.30, RT) at the Center for Microfluidics in Chemistry and Biology at the University of Toronto (Canada) with a Nikon A1 confocal microscope equipped with two camera systems, a Q imaging Retiga 2000R Fast 1394 camera and a Nikon confocal imaging system running Nikon NIS-Elements software ("Confocal-1"). All Figure 9 images were acquired on Confocal-1 with a Plan Fluar objective (Nikon, 40X, NA 1.30, oil immersion, RT). Figure 11 scanning electron microscope (SEM) images were acquired on a Phillips XL30 ESEM at the Department of Pathology and Laboratory Medicine, Mount Sinai Hospital. Samples were prepared according to imaging facility requirements. In brief, samples were fixed with glutaraldehyde and dehydrated stepwise to 100 % ethanol. After critical point drying, samples were gold coated with a low-vacuum sputter. Figure 11A and B confocal images were taken on a Leica DMIRE2 confocal microscope system, from here on referred as Confocal-2, with a HCX PL APO CS (Leica, 40X, NA 1.25, oil immersion, RT) objective and processed with Leica Software. Figure 11C and all confocal images from Figure 14 were taken on Confocal-1 with a Plan Fluar objective (Nikon, 40X, NA 1.30, oil immersion, RT). Figure 16 images of µ-CP were taken on Zeiss-1 with a Plan-Apochromat objective (Zeiss, 63X, NA 1.4, Oil-DIC, RT). Images in Figure 18A were taken on Zeiss-1 with a Fluar objective (Zeiss, 20X, NA 0.75, RT) and Figure 18C images were taken live with Zeiss-1 and a WN-Apochromat objective (Zeiss, 40X, NA, 1.0, RT). Figure 19 images are from a movie of the cellerator and LTBP-1 ECM (processed with MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). The movie was taken on a Axiovert 135M microscope (Zeiss, Germany) with a Hamamatsu C10600 ORCA-R2 camera and a Fluar
objective (Zeiss, 10X, NA 0.5, RT). Figure 20B images were taken on Confocal-1 with a Plan FLUAR objective (Nikon, 40X, NA 1.30, oil immersion, RT). Figure 17 images were taken on Zeiss-1 with a Fluor objective (Zeiss, 20X, NA 0.75, RT). All Zeiss microscopes operated with ZEN software (Zeiss, Germany).

Quantitative image analysis was performed using ImageJ (U. S. National Institutes of Health (NIH), Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2013) and customized macros. Figures were assembled with Adobe Photoshop CS5 (Adobe Systems). ED-A FN and LTBP-1 fibril count and density was determined from images taken from tissue slices and cell cultures. Briefly, single channel images from ECM stains were converted to 8 bit grey scale and thresholding was applied to remove background (unorganized ECM) depending on experimental conditions. Pixels with intensity higher than threshold were counted as particle events and density was analysed for events with a size larger than 2x2 pixels and 0-1 circularity.

3.3.7 Immunoprecipitation and Western blotting

For Western blots, vinculin, α-SMA, and vimentin were separated on 10% gels and FN, ED-A FN, FAK, GPF (reduced conditions) and LTBP-1 (non-reduced conditions) on 8% SDS-Page gels. Gels were transferred to nitrocellulose membranes using semi-dry transfer technique at 18 mAmps/gel, 20 V, for 16h or overnight. Protein membranes were blocked with 5% skim milk and primary antibodies were detected with fluorescently labeled anti-mouse-680 nm and anti-rabbit-800 nm IRDye® secondary antibodies (1:10000, LI-COR Biosciences, Cat#LIC-926-68020 and LIC-926-32211). Signals were detected with a LiCor Fx imaging system (LI-COR Biosciences).
3.3.8 TGF-β1 bioassay

Active TGF-β1 was quantified using TMLCs, which produce luciferase under control of the PAI-1 promoter in response to TGF-β1 (Abe et al., 1994). Co-culture or decellularized ECM experiments were performed by seeding TMLCs directly onto the first cell layer or DOC insoluble ECM. Fibroblast and myofibroblast contraction was then induced by 0.5 U/ml of thrombin for 1h with subsequent media change for 16h. TMLCs were lysed with cell culture lysis reagent (1:5, Promega, Cat#E1531) and assessed with a luciferase assay kit (Promega, Cat#E1500) using a luminometer (Centro LB, Berthold Technologies).

To assess TGF-β levels from culture supernatants, TMLCs (60,000 cells/cm²) were allowed to adhere for 4h before being subjected to conditioned media, native (active TGF-β) or heat-activated (total TGF-β) for 10 min at 80°C, respectively, for 1h. TMLCs were then grown in medium containing 1% FBS for 16h. TGF-β1 specific blocking antibody (5 ng/ml, R&D Systems, AF-101-NA) was used to confirm that TGF-β1 is the main TGF-β isoform activated in our cultures. If not stated otherwise, all results were corrected for TMLC baseline luciferase production in the absence of TGF-β1 and thrombin activation. In all conditions, active TGF-β1 was normalized to total TGF-β1.

3.3.9 ECM labeling with fluorescent microspheres

Red-fluorescence microspheres with 1 µm diameter (Cat# F8851, Life Technologies, Carlsbad, US) were coated with anti-LAP-1 antibody. ECM on HESR membranes was decellularized and stained for LTBP-1 and anti-mouse-IgG1-FITC as described. ECM was
decellularized with desoxycholate buffer (150mM NaCl, 50mM Tris-HCl, 1% NP-40, 0.5% sodium desoxycholate) treatment. In brief, cells were washed three times with PBS, ice-cold DOC buffer was added for 5-10 min at 4°C and cell debris was aspirated. Subsequent washes with PBS cleaned ECM of remaining debris. Non-strained and 1.9-fold strained ECM was incubated with an excess of microspheres for 5 min, followed by extensive washing to remove unbound spheres. Five random images of LTBP-1 fibril area and spheres were taken in three independent experiments and analysed for amount of LTBP-1 area and bound spheres.

3.3.10 Microcontact printing and cell adhesion assay

To visualize direct binding of cells to purified LTBP-1, we employed microcontact printing (Goffin et al., 2006). In brief, polydimethylsiloxane stamps exhibiting islet topographies with dimensions of 10 µm length, 1.5 µm width and 4 µm spacing, were coated with 0.2 µg/ml LTBP-1 or FN (Chemicon, Cat#FC010) for 1h. Protein prints were created on plastic coverslips and protein-free areas were passivated with poly-L-lysine-polyethylene glycol (PLL-g-PEG) for 10 min. Myofibroblasts were seeded on prints in serum-free media for 4h before being processed for immunostaining. For cell adhesion quantification, tissue culture plastic wells were coated with LTBP-1 (20 µg/ml) and hDMf were seeded for 4h in the presence of cyclic peptides antagonizing RGD (10 µM, GIBCO, Cat#12135-010) and control RGE (10 µM, GIBCO, Cat#12139-010, integrins αvβ3/αvβ5 (Cilengitide, EMD121974), αvβ3 integrin (EMD66203), and scrambled control (EMD135981) (Merck, Kirkland, QC, Canada).
Blocking antibodies were used directed against integrins β1 (10 µg/ml, Millipore, Cat#MAB1965), β3 (10 µg/ml, Millipore, Cat#MAB1976), and β5 (10 µg/ml, Millipore, Cat#MAB1961). Additional controls were RGE and human IgG (10 µM, Sigma Aldrich, Cat#I9135). Samples were rigorously washed three times before cells were fixed for quantification and staining.

3.3.11 ECM pre-strain assay

To strain decellularized ECM, we continuously expanded silicone culture substrates with a mechanical strain device (Cellerator, Cytomec GmbH, Spiez, Switzerland) and highly expandable silicone rubber culture membranes, chemically functionalized for cell adhesion and coated with covalently bound rat tail collagen type I at 10 µg/ml (BD Biosciences, Cat#354236) as previously described (Wipff et al., 2009) Fibroblastic cells were grown for 7d and removed with DOC to preserve ECM only (Wipff et al., 2007). Membranes and attached ECM were then uniaxially strained from 1.0 (non-strained) to 2.8-fold in 0.1% increments before hDMf were seeded and grown for 4h on the DOC-insoluble ECM. Finally, cell contraction was induced and TGF-β1 activation measured. In one series of experiments, TGF-β1 activation was measured during ECM pre-strain in the absence of any cells.

3.3.12 Cell contraction assay

Cell contractility was assessed using deformable silicone substrates as previously described (Godbout et al., 2013). In brief, polydimethylsiloxane (PDMS) substrates with a young’s modulus of 2 kPa were coated with 10 ng/ml FN.
Cells were seeded at a concentration of 2,500 cells/cm$^2$ and wrinkle formation on substrates was observed after 24h in culture.

Live phase contrast images were acquired with an inverted microscope (Axiovert135, Carl Zeiss, 40x objective) and analyzed using Image J software (NIH) by thresholding for phase-bright wrinkles and analyzing the surface area covered by identified particles in the resulting binary images. Relative contraction was expressed as the image area covered by wrinkles as previously described (Balestrini et al., 2012).

3.3.13 Statistical analysis

When applicable, data are presented as means ± standard deviation (SD). We assessed differences between groups with an analysis of variance (ANOVA) followed by a post-hoc Tukey’s multiple comparison test and we set the significance level at p=0.05. For experiments comparing fibroblasts versus myofibroblasts, we performed a two-tailed paired t-test. When applicable, differences were considered to be statistically significant and indicated with *p≤0.05, **p≤0.01 and ***p≤0.005. Error bars represent standard deviation.
3.4 Results

3.4.1 Myofibroblast differentiation leads to increased ECM organization and TGF-β1 activation

To test whether de novo formation of myofibroblasts and increased tissue stress in vivo are associated with higher fibrillar organization of ECM in general and LTBP-1 in particular, we employed a rat model of mechanically enhanced wound healing (Hinz et al., 2001b). The dermis of normal rat skin exhibited negligible levels of the fibronectin (FN) splice variant ED-A FN, LTBP-1, and no α-SMA-positive myofibroblasts (Figure 8). After dermal wounding, neo-expression of ED-A FN (day 3-4) preceded first appearance of LTBP-1 and myofibroblasts (day 6-7) in the granulation tissue with all proteins reaching peak expression at ay 9 (Figure 8A). The alignment of ECM fibrils in parallel to the skin surface moderately increased over time of normal healing (Figure 8A). In contrast, mechanically restraining the wound edges with splints accelerated ED-A FN, LTBP-1, and α-SMA expression by ~3 d and led to substantially higher fibril organization at any given time compared with normal wounds. Differences between normal and splinted wounds were most pronounced at 9d post-wounding, as shown by quantifying LTBP-1 fibril density by image analysis (Figure 8A).

Enhanced LTBP-1 organization correlated with the enhanced TGF-β1 downstream signalling (pSmad2/3 phosphorylation) and α-SMA expression reported in our previous studies using the same rat model (Hinz et al., 2001b; Wipff et al., 2007).
See legend next page
Figure 8: Changes in ECM composition and structure during myofibroblast differentiation.

(A) Granulation tissue sections of splinted and non-splinted wounds from female Wistar rats were stained for α-SMA (blue), ED-A FN (red) and LTBP-1 (green). Normal rat skin is compared to 3 day old non-splinted wounds. Additionally, the arrow indicates the region of granulation tissue that is compared from splinted and non-splinted wounds at day 3, 6 and 9. Image insert scale bars represent 10 µm. (B) hDF (control) were differentiated into hDMf using 2 ng/ml TGF-β1 for 5d in passage 1 (+TGF-β1) and used at passage 4 after 6d of growth. Cells were labelled for α-SMA (green) and F-actin (red), and for LTBP-1 (green), ED-A FN (red), and nuclei (blue). (C) Expression of ED-A FN, LTBP-1, vinculin, and α-SMA was determined by Western blotting of cells grown without (control) and with TGF-β1 and quantified as percentage of control normalized to vimentin as loading control. (D) The average density of fibril events per image field was quantified from 14 LTBP-1 stained images per 3 independent experiments. Active TGF-β1 was measured by directly co-culturing fibroblastic cells with TMLC reporter cells either after inducing cell contraction with thrombin (E) or without inducing cell contraction (F, baseline). Levels of active TGF-β1 are presented as percentage of total TGF-β1 determined from heat-activated ECM and cells (G). Graphs show mean values and standard deviations from at least three independent experiments (*p≤0.05, two-tailed paired Student’s t-test).

To discriminate the role of ECM strain in guiding fibrillar organization of LTBP-1 and availability of associated latent TGF-β1 we established controlled experimental conditions. High-contractile human dermal myofibroblasts (hDMf) were generated by treating primary human dermal fibroblasts (hDF) with active TGF-β1 once for 5d in passage 1. TGF-β1 used to differentiate hDMf was not detectable in passage 4 when hDMf were assessed experimentally (Figure 9A-B). In contrast to the small population of cells (7±5%) expressing the myofibroblast marker α-SMA in contractile stress fibres in standard culture conditions, the hDMf fraction represented 40±15% of the cell population even three passages after TGF-β1 stimulation (Figure 8B, Figure 9A-B). Higher protein expression levels of α-SMA in hDMf correlated with higher expression of LTBP-1 and ED-A FN (Figure 8B, C).
Figure 9: TGF-β1 effect on hDf and TMLC.

hDf were activated into hDMf by adding 2 ng/ml active TGF-β1 once for 5d to passage 1 cells. Over the following 3 passages, cell cultures were assessed (A) for active TGF-β1 in the supernatants and (B) myofibroblast marker protein expression. (C) HDMf under thrombin (Thr) stimulation release the TGF-β1 isoform as shown by adding anti-TGF-β1 antibody to the TMLC/hDMf co-cultures while inducing contraction. (D) Western blotting of ECM and media from hDMf cultures in the presence of thrombin, TRAP-6 (T-6) and scrambled TRAP-6 (scr T-6). (E) HDMf were used to produce ECM and removed using DOC; cell-free ECM was incubated with thrombin to control that no TGF-β1 is activated in the absence of contracting cells. (F) Active TGF-β1 released by hDMFs under thrombin, TRAP-6 or scrambled TRAP-6 stimulation. (G) Immunofluorescence staining of ECM and media from hDMf cultures in the presence of thrombin, TRAP-6 (T-6) and scrambled TRAP-6 (scr T-6). ED-A FN (red), LTBP-1 (green) and DAPI (blue). Scale bar: 25 µm.
Organization of LTBP-1 and co-localization with ED-A FN in the myofibroblast ECM was 3-fold higher than in the ECM produced by low contractile hDf, as quantified by LTBP-1 density in fibrils (Figure 8B, D). To compare the potential of hDf and hDMf to activate TGF-β from their differently organized ECM, we induced cell contraction with thrombin. Active TGF-β was measured using directly co-cultured transformed mink lung epithelial reporter cells (TMLCs) and normalized to total levels of TGF-β measured in heat-activated cell/ECM lysates. TMLC reporting was abolished by adding TGF-β1-blocking antibodies to contraction-stimulated hDMf/TMLC co-cultures (Figure 9C), identifying TGF-β1 as the main isoform in hDMf cultures. Low concentrations of thrombin (0.5 U/ml) exerted no proteolytic action on either ECM or latent TGF-β1 (Figure 9D-E) confirming previous controls with other fibroblast sources (Sarrazy et al., 2014; Wipff et al., 2007). Thrombin-induced cell contraction and ECM deformation resulted in mechanical TGF-β1 activation similar to inducing cell contraction with thrombin receptor activating peptide-6 (TRAP-6), bearing no catalytic activity (Figure 9F-G). Contraction-induced active TGF-β1 levels were 3-fold higher in hDMf (36% of total TGF-β) than in hDf (11%) cultures (Figure 8E). Baseline levels of active TGF-β without addition of thrombin were low in both cell types (≤2%) (Figure 8F). Total levels of TGF-β were only moderately higher in hDMf than in 6d-old hDf cultures (Figure 8G).
Figure 10: ECM maturation and pre-remodeling affects TGF-β1 activation by myofibroblasts.

HDMf were grown for 2, 3, and 6d and lysates were used for Western blotting with vimentin as loading control. (B) hDF- or hDMf-derived ECMs were decellularized using DOC and heat-activated before total amounts of TGF-β1 were determined by luciferase reporter assay. (C) HDMf were seeded onto different pre-produced ECMs, stimulated to contract with thrombin, and active TGF-β1 was measured as percentage of total TGF-β1. (D) HDMf were transfected with LTBP-1-EGFP and stained for LTBP-1 (red) and EGFP (green) after 12 d. (E) EGFP (green), ED-A FN (red) and nuclei (blue) were stained in hDMf cultures after 2, 3 and 6 d. Bottom rows show magnified ECM regions. (F) The average density of LTBP-1-EGFP fibrils was quantified from stained images. Scale Bars: D, 150 µm; E, 25 µm, close-up: 5 µm. Graphs show mean values and standard deviations from at least three independent experiments (*p≤0.05 and ***p≤0.005 using ANOVA followed by a post-hoc Tukey’s multiple comparison test).
3.4.2 Mechanical activation of TGF-β1 increases with increasing maturation of the ECM

Next, we tested whether the higher levels of active TGF-β1 in hDMf cultures are related to ECM properties rather than to the TGF-β1-activating fibroblast phenotype. HDF and hDMf were cultured to produce latent TGF-β1-containing ECM for 2d, 3d, and 6d. LTBP-1 expression in hDMf cultures was detectable by 3d and increased by 6d; FN (total and ED-A FN) was detected already after 2d and increased only moderately over culture time (Figure 10A). LTBP-1 and FN expression levels in HDF cultures followed the same time course, but were lower than in hDMf cultures (data not shown and Figure 8). The differently produced and matured ECMS were then decellularized using desoxycholate (DOC) and total TGF-β1 levels were measured. In the 3d and 6d-old decellularized ECM, total TGF-β1 levels were similar and only moderately higher (1.25-fold) in hDMf than in HDF cultures (Figure 10B). However, when hDMFs were re-seeded onto all decellularized ECMS and induced to contract, an approximately 2-fold increase in active TGF-β1 (% of total) was observed on the 6d mature versus the 3d immature ECM (Figure 10C). Moreover, ~2-fold more TGF-β1 was released by re-seeded hDMf from hDMf-remodelled ECM than from HDF-remodelled ECM of the same ‘age’ (Figure 10C). To relate TGF-β1 activation with LTBP-1 organization in the ECM, we transfected enhanced green fluorescent protein (EGFP)-tagged LTBP-1 into hDMFs. LTBP-1-EGFP co-localized with endogenous LTBP-1 in the ECM (Figure 10D). In 2d-old ECM, LTBP-1 was almost exclusively localized in patches at the substrate surface that became increasingly organized into ED-A FN-containing fibrils after 3d and 6d culture (Figure 10E, F).
Figure 11: Myofibroblasts are more efficient in LTBP-1 ECM organization than fibroblasts.

LTBP-1 substrates were produced by using (A) LTBP-1-EGFP transfected HEK293 cells that were grown for 7 d, (B) ECM obtained after DOC treatment of LTBP-1-EGFP transfected HEK293 cells that were grown for 7 d, and (C) substrates coated with LTBP-1-EGFP purified from supernatants of LTBP-1-EGFP transfected HEK293 cells. The resulting ECM was used for scanning electron microscopy (SEM) and as substrates for hDf and hDMf. Fibroblastic cells were stained after 2 days growth for EGFP (green) and ED-A FN (red). Scale bars: SEM images, 5 µm and 500 nm for insets; immunofluorescence images, 20 µm. LTBP-1-EGFP fibrils were quantified by image analysis from at least 5 images per three independent experiments to calculate mean values and standard deviations (***p<0.005 using ANOVA followed by a post-hoc Tukey’s multiple comparison test).
Figure 12: Purification of LTBP-1.

LTBP-1-EGFP was purified from conditioned serum-free medium of LTBP-1-EGFP overexpressing HEK293 cells, using the 6xHis-tag on LTBP-EGFP. Coomassie gel and Western Blot assays confirmed presences of LTBP-1 in elution fraction 1.

These results indicate that the efficacy of mechanical activation of TGF-β1 correlates with the level of FN fibril formation and organization of the ECM in addition to the acute contractile state of the fibroblastic cells.

3.4.3 Myofibroblasts incorporate LTBP-1 into strained ECM fibrils in an integrin-mediated and actin-myosin-dependent process

Thus far, our results suggest that a higher degree of ECM organization contributes to the increased ability of hDMf to generate active TGF-β1 compared with hDf.
Figure 13: LTBP-1 organization by myofibroblasts.

(A) LTBP-1-EGFP overexpressing HEK293 cells were used to produce LTBP-1-containing ECM. After extraction of HEK293 using DOC, the remaining DOC-insoluble ECM was used as substrate for hDMfs. HDMFs were allowed to adhere for 4h before video-recording ECM organization for 220 min with image intervals of 15 s. The EGFP-tag on LTBP-1 was used to visualize fibril formation at a wavelength of 488 nm. Scale bar: 20 µm. (B) To knock-down FN, hDMFs were transfected with siRNAs directed against the FN gene; controls were mock transfected without siRNA and non-targeting (NT) RNA sequences. (B) HDMf and HDF were grown on LTBP-1-EGFP coated substrates for 2d in the absence and presence of excess FN (100 µg/cm²). Fibril counts were quantified from LTBP-1-EGFP by image analysis. Graph shows mean values and standard deviations from at least three independent experiments (*p≤0.05, two-tailed paired Student's t-test).

To determine the remodelling capacities of hDMf and HDF independently of LTBP-1/ECM secretion, we pre-produced LTBP-1 containing ECM in three different ways (Figure 11). First, human embryonic kidney-293 (HEK293) cells were stably transfected with LTBP-1-EGFP and used to generate an ECM enriched in EGFP-tagged LTPB-1. The HEK293 cells were not able to organize FN or the secreted LTBP-1-EGFP into fibrils (Figure 11A).
Figure 14: FN mediates LTBP-1 fibril formation and incorporation.

Substrates were coated with purified LTBP-1-EGFP and used to culture (A) hDMf transfected with siRNA against FN (siFN) or non-related control RNA (siNT), (B) FN<sup>-/-</sup> MEF or wild-type (WT) MEF. (C) hDF and hDMf were seeded onto substrates coated with LTBP-1-EGFP alone or LTBP-1-EGFP mixed with an excess of FN (100 µg/cm<sup>2</sup>) and grown for 4 h. (D) HDMf were pre-grown for 7d before purified LTBP-1-EGFP was added to the culture medium for additional 24h in the presence or absence of 20 µM blebbistatin (blebbi). Experiments were performed in FN-depleted culture medium. All samples were stained for EGFP (green), FN (red) and nuclei (blue). Scale bars: 25 µm. LTBP-1-EGFP fibrils (A-D) and staining intensity (E) were quantified by image analysis from at least five images per three independent experiments to calculate mean values and standard deviations (*p≤0.05 and ***p≤0.005 using ANOVA followed by a post-hoc Tukey’s multiple comparison test).
Second, at variance with the first setup, the ECM-producing HEK293 cells were removed using DOC, leaving behind LTBP-1-EGFP patches on the substrate surface (Figure 11B). Third, LTBP-1-EGFP was purified from HEK293 cell supernatants and used to coat culture substrates (Figure 11C, Figure 12). Scanning electron microscopy demonstrated the ultrastructurally different ECMs (Figure 11). HDMf and hDf were then added to process the pre-fabricated ECM in FN-depleted medium for 2d (Figure 13A). During this time, the endogenous production of LTBP-1 was negligible (Figure 10). Organization of the supplied LTBP-1-EGFP into ECM fibrils was significantly higher in hDMf than in hDf cultures in all three experimental models (Figure 11).

LTBP-1 incorporation into the ECM was previously shown to depend on FN secretion (Dallas et al., 2000; Massam-Wu et al., 2010; Taipale et al., 1996; Todorovic and Rifkin, 2012; Zilberberg et al., 2012a) and binding of LTBP-1 to FN is essential for integrin-mediated activation of TGF-β1 (Annes et al., 2004a). To test whether fibroblastic cells organize LTBP-1 directly or indirectly by hitchhiking on FN fibril formation, we knocked-down FN in hDMf using specific siRNA (Figure 13B). FN-deficient hDMf were able to attach and spread onto LTBP-1 coated substrates but failed to form LTBP-1 fibrils (Figure 14A). Similar results were obtained using FN−/− mouse embryonic fibroblasts (MEF) (Figure 14B), confirming that the presence of FN is indeed crucial for LTBP-1 fibril formation. HDMf produced higher amounts of FN than hDf during LTBP-1 fibril formation (Figure 10, Figure 11).
To evaluate if higher FN quantities accounted for the higher efficiency of hDMf to produce LTBP-1-containing fibrils, we restricted the culture time of hDf (Figure 14C) and hDMf (Figure 14D) to 4h on LTBP-1-EGFP-coated substrates in FN-free culture medium. Quantification of LTBP-1-EGFP immunostaining and videomicroscopy analysis demonstrated that hDMf assembled LTBP-1-EGFP almost instantaneously into fibrils, which were always positive for cell-derived ED-A FN. In accordance with previous observations, the LTBP-1 fibril count was higher for hDMf than for hDf (Figure 14C, D, Figure 13C). Importantly, hDMf also incorporated ~2-fold more LTBP-1-EGFP into fibrils than hDf when cultures were supplemented with an excess of plasma FN (100 µg/cm²), either added to the medium (unpublished data) or adsorbed onto the culture surface together with LTBP-1-EGFP (Figure 14C, D, Figure 13C).

Previous studies have demonstrated that LTBP-1 is increasingly transferred from FN to fibrillin-1 during fibroblast culture ECM maturation (Zilberberg et al., 2012a). In our fibroblast cultures, FN and fibrillin-1 strongly co-localized up to 6d of cell culture (Figure 15A). To test whether fibrillin-1 is important in mechanical priming of the ECM for TGF-β1 activation, we produced ECM using mouse dermal fibroblasts bearing a fibrillin-1 mutation in LTBP-1 binding. Fibrillin-1 mutant murine fibroblasts produced and organized LTBP-1 (Figure 15C); however, the amount of total TGF-β1 in the ECM was ~4-times lower than in wild-type cultures. Consequently, pre-organization did not change the amount of TGF-β1 activated by hDMfs from fibrillin-1 mutant ECM whereas pre-organization improved TGF-β1 activation by 1.5-fold from wild-type mouse fibroblast ECM (Figure 15D).
Figure 15: Importance of fibrillin-1 in LTBP-1 fibril formation.

(A) Myofibroblasts were cultured for 6d and co-immunostained for fibrillin-1 (green), ED-A FN (red) and nuclei (blue). (B) Wild-type (WT) and fibrillin-1 C1039G/+ mutant mouse dermal myofibroblasts (mDMf) cultures were grown for 7d and analysed by Western blotting. (C) WT and fibrillin-1 C1039G/+ mutant mDMf were grown on purified LTBP-1-EGFP for 2d and stained for FN (red), GFP (green) and nuclei (blue) to quantify the number of LTBP-1 fibrils by image analysis from at least three images per three independent experiments. (D) WT and fibrillin-1 C1039G/+ mutant mDMf grown on relaxed highly expandable silicone membranes were removed after 6d using DOC. The decellularized ECM was then strained in the absence of cells by 1.9-fold and hDMf were seeded onto non-strained and pre-strained decellularized ECM. Cell contraction was induced using thrombin and release of active TGF-β1 was quantified as percentage of total TGF-β1. Graph shows mean values and standard deviations from at least three independent experiments (**p≤0.01, ***p≤0.005, two-tailed paired Student’s t-test). Scale bars: 20 µm.
We next investigated the incorporation of LTBP-1 into an established ECM network by adding purified, soluble LTBP-1-EGFP to hDMf that were pre-cultured for 7d to produce their own ECM (Figure 14E). The supplemented LTBP-1-EGFP preferentially co-localized with pre-existing ED-A FN fibrils (Figure 14E, inset) and the incorporation process was abolished in the presence of 20 µM blebbistatin (Figure 14E). Collectively, these results show that: 1) The higher efficacy of hDMf to form LTBP-1-containing fibrils is not due to higher production of FN, 2) hDMf incorporate exogenous LTBP-1 into the ECM during FN fibrillogenesis; 3) This process is cell-mediated and dependent on actin-myosin activity.

Because human LTBP-1 contains an RGD consensus site, it is conceivable that αv integrins participate in LTBP-1 incorporation into ECM fibrils and cell attachment to LTBP-1-coated substrates. To identify the integrin putatively mediating adhesion to LTBP-1, we generated microcontact printed islet arrays of purified LTBP-1-EGFP with typical focal adhesion features (10x4x1.5 µm).
Figure 16: Integrins mediate binding of myofibroblasts to LTBP-1.

Arrays of islet with typical focal adhesion features (10x4x1.5 µm) were microcontact printed using purified LTBP-1-EGFP (A) or plasma FN as control (B). After attachment for 4h to microcontact printed arrays in the absence of serum, hDMS were immunostained for the printed protein (red, false colored for LTBP-1-EGFP), vinculin, and integrin subunits β1, β3 and β5 (green). (C) HDMfs were seeded onto LTBP-1-EGFP coated substrates in the presence of blocking antibodies or cyclic peptides against integrin subunits β1, β3, β5 or RGD-site. Focal adhesion formation was visualized with vinculin (green) and nuclei with DAPI (blue). (D) The number of adherent hDMS on LTBP-1-EGFP coated substrates was quantified by image analysis. Scale bars represent 20 µm an in inserts 5 µm.
Figure 17: ECM immunofluorescence staining of wild-type, filamin A knock-down and integrin β1−/− MEF.

Wild-type MEF, filamin A knock-down MEF and integrin β1−/− MEF were grown for 6d and stained for FN (red), LTBP-1 (green) and nuclei (blue).

HDMf adhered specifically to the printed LTBP-1 at sites of vinculin-positive focal adhesions when seeded for 4h in serum-free medium (Figure 16A). Focal adhesions forming on LTBP-1 islets contained β1 integrin, to lesser extent β3 integrin, and no integrin β5. Control prints using FN as a ligand demonstrated that all tested integrins were expressed in hDMf and localized to focal adhesions (Figure 16B).

Next, we seeded hDMf onto fully LTBP-1 coated substrates for 4h in the presence of specific integrin-blocking antibodies and different RGD peptides (Figure 16C). Focal adhesion formation and cell spreading were reduced by all integrin blocking antibodies in order from strongest to weakest: β1, β3, and β5 integrin. All competitive integrin blocking peptides, but not controls, inhibited focal adhesion formation and cell spreading on LTBP-1 (Figure 16C).
See legend next page
Figure 18: ECM disorganization suppressed TGF-β1 activation.

FAK\(^{-/-}\) MEF and wild-type (WT) were cultured for 6 d. (A) ECM production was assessed by immunofluorescence staining for FN (green) and nuclei (blue) and the average density of FN fibrils was calculated from six images per three independent experiments. (B) Culture lysates were immunoblotted using vimentin and GAPDH as loading controls. (C) Cells were seeded for 1d onto silicone substrates and wrinkle formation due to substrate deformation was quantified from phase contrast images. (D) After 6d culture, MEF were removed using DOC and total TGF-β1 was measured after heating the remaining ECM. (E) HDMF were seeded onto the decellularized MEF ECM; active TGF-β1 levels were measured as percentage of total TGF-β1 without (baseline) and with thrombin-induced contraction. (F) TMLC alone were seeded onto decellularized ECM produced by MEF WT and FAK\(^{-/-}\) and stimulated for 1h with 0, 0.03, 0.06, 0.12, 0.25, 0.50, and 1.0 ng/ml of active TGF-β1. Cells were further processed and assessed for luciferase production (luminescence). The data shown are from a single representative experiment and experiment was completed once with three independent measurements. (G) Decellularized MEF ECM was incubated for 1h with magnetic beads coated with BSA or anti-LAP antibody before magnetic force was applied and active TGF-β1 was measured in the supernatant. Graphs show mean values and standard deviations from at least three independent experiments (***p≤0.005 using ANOVA followed by a post-hoc Tukey’s multiple comparison test).

Strongest blocking was achieved with RGD as confirmed by quantifying the number of adherent hDMFs on LTBP-1 coated substrates (Figure 16D). These results indicate that hDMF bind directly to the RGD sequence in LTBP-1 via integrins that possibly aid the active organization of LTBP-1 into ECM fibrils in a cell tension-dependent manner. Of the RGD-recognizing integrins expressed in hDMf, β5 integrin seem to bind weakest by far.

3.4.4 Incorporation of LTBP-1 into disorganized ECM impairs mechanical activation of TGF-β1

To further investigate how the higher pre-organization of the hDMf ECM affects the efficacy of TGF-β1 activation, we generated ECM with MEF exhibiting cell-ECM protein interaction defects. MEF derived from mice deficient for focal adhesion kinase (FAK\(^{-/-}\)) (Rajshankar et al., 2012) and β1 integrin (β1\(^{-/-}\)) (Fassler and Meyer, 1995), and MEF stably knocked-down for filamin A (Kim et al., 2008), all produced disorganized ECM compared to wild-type MEF after 6d of culture (Figure 17, Figure 18A).
We continued with FAK\textsuperscript{-/-} MEF that produced a disorganized ECM with low FN fibril density (Figure 18A), but expressed LTBP-1 and FN at levels similar to those of wild-type MEF (Figure 18B). This phenotype confirmed previous studies relating poor FN fibrillogenesis to the central role of FAK in fibrillar adhesion formation (Ilic et al., 2004). In addition to these studies, we herein show that FAK\textsuperscript{-/-} MEF exhibit significantly lower expression levels of α-SMA (Figure 18B) and exert lower contraction forces to deformable culture substrates (Figure 18C) as compared to wild-type MEF. Despite the organizational differences, the amounts of total TGF-β1 in the ECM of DOC-treated FAK\textsuperscript{-/-} and wild-type MEF were similar (D). In contrast, active TGF-β1 levels differed significantly when hDMf were seeded onto the decellularized murine ECMs and induced to contract. HDMf contraction activated 50% of the total TGF-β1 stored in the ECM from wild-type MEF but only 20% from the FAK\textsuperscript{-/-} ECM (Figure 18E). Baseline TGF-β1 activation in the absence of contraction was low (~15%) and comparable on both ECM types (Figure 18E).

Addition of the contraction agonist thrombin to decellularized MEF ECM in the absence of hDMf did not release any TGF-β1, confirming the complete removal of MEF and the specific action of thrombin on hDMf contraction (unpublished data). Control experiments performed with active TGF-β1-stimulated TMLC verified that the reporter cell activity did not depend on the organization level of their ECM substrate (Figure 18).

Although attachment and spreading of hDMfs was similar on the different MEF-derived ECMs, ECM-induced phenotypic changes in hDMf may have affected TGF-β1 activation. To exclude this possibility, we mechanically released active TGF-β1 using a cell-free assay (Figure 18F) (Buscemi et al., 2011b).
See legend next page
Figure 19: Pre-straining LTBP-1-containing ECM enhances subsequent TGF-β1 release.

HDMf grown on relaxed highly expandable silicone membranes were removed after 6d using DOC. Scale bar represents 20 µm and on magnified images 4 µm. (A) The cell-free DOC-insoluble ECM was stained without fixation for LTBP-1 and visualized during membrane expansion from relaxed (1.0-fold) to 2.8-fold strain. Selected fibrils (box) were magnified and followed in incremental steps of 0.2-fold membrane strain. (B) Illustration of the strain device opening and membrane expansion. Strain of LTBP-1-containing fibrils was measured as fold length change compared to initial length and plotted against membrane surface expansion. The data shown are from a single representative experiment out of three repeats. (C) HDMf were seeded onto non-strained (1.0-fold) and pre-strained decellularized ECM (up to 2.2-fold). Cell contraction was induced in every condition using thrombin and TRAP-6 in select conditions and release of active TGF-β1 was quantified as percentage of total TGF-β1. Western blotting was performed for phospho- and total Smad2 in subsequently lysed myofibroblasts. (D) The decellularized ECM produced by either hDMFs or hDF was strained in the absence of cells by expanding the membrane up to 2.8-fold and active TGF-β1 released into the supernatant was measured at every 0.1-fold increment. (E) Decellularized ECM labelled for LTBP-1 (red) and green-fluorescent microspheres at strains of 1.0- and 2.8-fold. White arrows indicate microspheres bound to LTBP-1 fibrils. The number of microspheres in the image field was quantified and normalized to the area covered by LTBP-1 fibrils. (F) Active TGF-β1 (1 ng/ml) was added for 1h to cell-free hDMf-derived ECM that was either non-strained (1.0) or strained 1.9-fold. Samples were rigorously washed 3-times, ECM subsequently lysed and levels of ECM-contained TGF-β1 was measured using TMLC. Graph shows mean values and standard deviations from at least three independent experiments.

Decellularized MEF-derived ECM was incubated with anti-LAP coated ferromagnetic beads for 1h before force was applied through a magnetic field and active TGF-β1 was measured (Figure 18G). Force-induced release of active TGF-β1 from FAK<sup>-/-</sup> MEF ECM was low (10% of total TGF-β1) and not different from no-force and BSA-coated bead controls (Figure 18G). Conversely, pulling magnetic beads coated with anti-LAP antibody resulted in ~3-fold higher release of active TGF-β1 from wild-type MEF ECM as compared to FAK<sup>-/-</sup> MEF ECM (Figure 18G). Collectively, these data demonstrated that mechanical activation of TGF-β1 from pre-organized and mature ECM is more efficient than from disordered ECM.
3.4.5 Mechanical loading of LTBP-1 fibrils enhances TGF-β1 release

Fibroblast-mediated ECM maturation involves straining of secreted fibrils, bundling, and proteolytic remodelling. To test whether pre-straining the ECM alone would facilitate TGF-β1 activation, we used mechanical strain device and highly expandable silicone culture membranes allowing up to 8-fold strain (Majd et al., 2011; Rosenzweig et al., 2012). The device was used to simulate and accelerate strain in the ECM that is induced by cells after several days or weeks in contrast to conventional strain devices and membranes that allow a maximum linear expansion of up to 1.3-fold, corresponding to length change associated with single cell contractions (Wipff et al., 2009). HDF were grown on non-strained membranes (1.0-fold) to produce ECM and removed after 6d using DOC. The remaining LTBP-1/TGF-β1-rich ECM was then strained in the absence of any cells. Immunofluorescence video microscopy of live-strained LTBP-1 revealed that, starting after a pre-strain of 1.4-fold, LTBP-1-containing fibrils straightened linearly with membrane strain to ~2.0-fold of their initial length at a membrane strain of 2.8-fold (Figure 19A-B). At strains larger than 3.0-fold, ECM was detaching from the membrane and thus not investigated (unpublished data). HDMf were then seeded on the differently strained ECMs and induced to contract with thrombin and TRAP-6 in select conditions (Figure 19C). HDMf contraction activated 7% of total TGF-β1 at a pre-strain of 1.7-fold, 13% from 1.8-fold pre-strained ECM, and 61% from 1.9-fold pre-strained ECM (Figure 19C). Western blotting for phospho-Smad2 in subsequently lysed hDMf confirmed that increasing pre-strain in the ECM resulted in increasing level of active TGF-β1 and downstream signalling (Figure 19).
Because no TGF-β1 was activated by contraction of re-seeded hDMf from hDf ECM that has been pre-strained by more than 2.0-fold, we hypothesized that larger strains can lead to release of TGF-β1 from the latent complex even in the absence of counteracting cell integrins. We tested this possibility by producing LTBP-1 ECM using hDf and hDMf and removing the cells after 6d with DOC. The cell-free ECM was then strained from 1.0-2.8-fold. Active TGF-β1 levels in the supernatant were measured after each incremental strain of 0.1-fold (Figure 19D). After strains of 2.0, 2.1, and 2.2, respectively, 11%, 23%, and 28% of the total TGF-β1 was activated and released from hDMf ECM. In contrast, release of active TGF-β1 from hDf ECM did not occur at strains lower than 2.4-fold. Collectively, these results indicate that 1) pre-straining ECM alone is sufficient to increase the release of active TGF-β1 by subsequent hDMf contraction; 2) high strain is able to mechanically open the latent complex for active TGF-β1 release even in the absence of cells, and 3) hDMf ECM reaches the TGF-β1 activation threshold at lower strains than hDf ECM. Alternatively, it is possible that strained ECM fibrils reveal a higher number of latent TGF-β1 complexes that are available for integrin binding. To test this possibility, we produced 1.0-fold (no strain) and 1.9-fold pre-strained hDf ECM as described above that was incubated with anti-LAP antibody-coated fluorescent microspheres for 5 min. After rigorous washing the number of beads remaining adherent per LTBP-1 fibril area in the image field was similar in both conditions, demonstrating that strain did not reveal ‘cryptic’ LAP (Figure 19E). We further excluded that active TGF-β1 absorbs differently to ECM under different strain conditions (Figure 19F). Therefore, we conclude that strain introduced into LTBP-1 fibrils primes the latent complex for TGF-β1 by myofibroblasts contraction.
3.5 Discussion Chapter 3

In a previous publication we showed that fibroblasts directly activate TGF-β1 by exerting forces to the latent complex and that cultured rat lung myofibroblasts release more active TGF-β1 from their ECM than fibroblasts (Wipff et al., 2007). These findings were explained with the higher contractile activity of myofibroblasts over fibroblasts (Hinz et al., 2001a).

However, recent structural and single molecule force spectroscopy studies have indicated that forces as low as 40 pN, corresponding to the action of myosin II motors, are sufficient to induce conformational changes in LAP that will lead to the release of TGF-β1 (Buscemi et al., 2011b; Shi et al., 2011). Consistently, low contractile epithelial cells are also able to activate TGF-β1 through integrin-mediated force transduction (Giacomini et al., 2012). One possible explanation for this apparent contradiction is that the efficacy of force-mediated TGF-β1 activation depends on the mechanical properties of the myofibroblast ECM that are established before the actual activation step occurs. Here, we demonstrate in vitro and in vivo that myofibroblasts deposit similar levels of latent TGF-β1 into the ECM, but organize the LTBP-1 into straighter and denser fibrils as compared to fibroblasts. By offering myofibroblasts latent TGF-β1-containing ECM of identical composition but different pre-strain, we revealed a clear dependence of TGF-β1 activation on the level of ECM organization at the time of force exertion to the latent complex. We propose a model where cell-mediated remodelling leads to gradual straining of LTBP-1/TGF-β1 containing ECM fibrils, analogous to loading a mechanical spring (Figure 20).
Figure 20: ECM pre-strain generated by myofibroblast contraction affects TGF-β1 activation.

Fibroblasts secrete ECM that is rich in FN (green) and the large latent complex of TGF-β1 (LTBP-1, LAP, and TGF-β1). Fibroblast-to-myofibroblast differentiation occurs during physiological (normal wound healing) and pathological (fibrosis) tissue remodelling. Myofibroblasts are characterized by α-SMA positive stress fibres (red) enabling these cells to exert high contractile activity and forces transmitted to the ECM at sites of integrins. The gradual straitening and straining of ECM fibrils, containing FN and LTBP-1, primes the latent TGF-β1 complex for subsequent activation. At sufficient pre-strain, minimal additional length changes in the ECM (i.e., small contractions) will be sufficient to release active TGF-β1 by inducing a conformational change in LAP. Hence, the mechanical pre-loading of the ECM determines the trigger point for TGF-β1 activation driving the vicious loop of myofibroblast self-activation.

Mechanical pre-loading of the ECM will have important implications for the availability of pro-fibrotic TGF-β1 during normal tissue repair and development of fibrosis. Organ fibrosis comprises the excessive secretion and contraction of ECM by fibroblastic cells and augments tissue stiffness (Henderson and Sheppard, 2013; Hinz, 2012; Klingberg et al., 2013; Wynn, 2008).
Others and we have previously shown that growth on culture substrates with low elastic modulus reduces TGF-β1 activation by cell contraction (Giacomini et al., 2012; Wipff et al., 2007). These findings led to the conclusion that the ECM has to be sufficiently stiff to resist the cell forces needed to release TGF-β1 from its LAP straitjacket.

Consistently, activation of TGF-β1 from the ECM depends on binding of LAP to LTBP-1 which in turn has to be linked to the ECM (Annes et al., 2004a; Fontana et al., 2005; Taipale et al., 1994). However, the view of stiffness-controlled TGF-β1 activation may be oversimplified in light of our results showing that pre-straining latent TGF-β1-containing ECM on silicone membranes with unchanged elastic modulus was sufficient to enhance TGF-β1 activation by subsequent cell contraction. Nevertheless, the bulk stiffness of the material may have a secondary effect by determining the organization state of the cell-derived ECM by regulating the remodelling activity of the adhering cells prior to TGF-β1 activation (Godbout et al., 2013).

In contrast to elastic cell culture polymers, the ECM of normal and fibrotic connective tissues is subject to strain-stiffening (Discher et al., 2009a; Storm et al., 2005). Our results suggest that cell remodelling strain-stiffens LTBP-1-containing ECM and thereby primes latent TGF-β1 for subsequent activation. The pathological remodelling and stiffening of scar tissue can be understood as the overall outcome of subcellular contractions with small length changes (~400 nm) but repetitive occurrence (~40 contractions/h) (Follonier Castella et al., 2010a).
Because such subcellular contractions theoretically accumulate to ~2.5 mm per week in vitro, it may take weeks, months, or years for fibrotic scars to mature (Follonier Castella et al., 2010b; Tomasek et al., 2002). ECM fibril straightening is greatly accelerated in cultures of myofibroblasts that are contracting with higher frequency and amplitude than fibroblasts (Follonier Castella et al., 2010a). In our experiments, mechanical release of TGF-β1 was dependent on the extent of ECM pre-remodelling and thus more efficient from a myofibroblast-produced ECM than from fibroblast-produced ECM. The fact that induced acute myofibroblast contraction results in larger absolute length changes than fibroblast contraction is likely responsible for their higher capacity to activate TGF-β1 from the same pre-formed ECM.

Pre-straining ECM on highly expandable culture membranes demonstrated that even the quasi two-dimensional ECM of cultured fibroblasts provides sufficient buffer (slack) to allow ~1.4-fold length changes before fibrils are visibly engaged and it requires ~2.8-fold strain to liberate TGF-β1 in the absence of cells. By contrast myofibroblast ECM is ~25% more pre-strained than the ECM of fibroblasts in our culture conditions as extrapolated from the release of TGF-β1 at lower strain (2.0-fold). It remains elusive whether TGF-β1 release in the absence of any cells was due to ECM damage by ‘overstrain’ or whether a physiological mechanism is conceivable that allows stress liberation of TGF-β1 without integrin pulling on the RGD site of LAP. A similar, cell cytoskeleton-independent effect was reported for strained tendon which is characterized by highly organized and strained collagen fibrils (Maeda et al., 2011).
It has been proposed that normal ECM strain in the tendon releases physiological levels of TGF-β1 that regulate tenocyte function; tendon injury and loss of the mechano-protective collagen structure was shown to result in dramatically increased active TGF-β1 levels (Maeda et al., 2011). Linking the activation of latent TGF-β1 to the organization state of the ECM will provide a mechanical threshold to generate and/or sustain myofibroblasts which develop their contractile activity by expressing α-SMA in response to TGF-β1 (Hinz et al., 2001a). In the poorly organized but latent TGF-β1-rich provisional ECM established after acute injury (Brunner and Blakytny, 2004), TGF-β1 activation by cell traction will be inefficient and myofibroblasts will not develop. In a sufficiently pre-strained ECM, even the low contractile forces exerted by migrating fibroblastic cells will be sufficient to promote latent TGF-β1 activation.

This model can answer the classical hen-and-egg question of whether ECM stiffening must occur first to induce myofibroblast differentiation or whether myofibroblast contraction must occur first to stiffen the ECM. Importantly, secretion of latent TGF-β1 by myofibroblasts into an already straightened fibrotic ECM will provide a much faster trigger for TGF-β1 activation than required during fibrosis development. This property may at least partly explain the fact that decellularized ECM derived from fibrotic but not normal lung tissue instructs de novo seeded cells to become fibrotic even in the absence of exogenous TGF-β1 (Booth et al., 2012a). It becomes indeed increasingly accepted that the biochemical and biophysical properties of fibrotic ECM provide sufficient cues to drive resident and recruited cells into a disease state (Berry et al., 2006; Shimbori et al., 2013).
The question remains whether enhanced activation of TGF-β1 requires pre-strain in LTBP-1 itself or whether LTBP-1 passively ‘piggy-backs’ on overall ECM remodelling. To date there is no evidence for formation of LTBP-1 exclusive fibrils. Our results show that LTBP-1 is secreted independently of FN but its fibrillar organization appears to depend on the presence of FN in the ECM. This interdependence was particularly evident in fibroblasts that overexpress LTBP-1 under a constitutively active promoter; in these cells the physiological sequence of FN preceding LTBP-1 secretion (Dallas et al., 2005; Koli et al., 2005) is uncoupled.

Our observations are consistent with the idea of FN acting as master-template in immature ECM for the subsequent recruitment and organization of other ECM proteins, including LTBPs (Dallas et al., 2005; Koli et al., 2005), fibrillin-1 (Chaudhry et al., 2007; Isogai et al., 2003; Ono et al., 2009; Sabatier et al., 2009; Sabatier et al., 2013), fibulin-1 (Godyna et al., 1995), collagen (Isogai et al., 2003; Velling et al., 2002) as well as the indirect role of heparan sulfate proteoglycans in LTBP-1 to FN binding (Chen et al., 2007). It is still unclear whether fibrillin-1 is another essential component to mechanically prime the ECM for subsequent TGF-β1 activation. The ECM produced by fibrillin-1 mutant murine fibroblasts in our experiments was too low in total TGF-β1 to exhibit clear differences in TGF-β1 activation before and after pre-strain. Further, fibrillin-1 and FN largely colocalize in 6d fibroblast cultures, it is reasonable to assume that the mechanism of ECM strain controlling TGF-β1 activation is principally independent from the molecular nature of the LTBP-1 binding protein provided that cell force can be transmitted.
Our new finding that fibroblastic cells incorporate non-endogenous purified LTBP-1 into pre-existing or developing FN ECM show that LTBP-1 fibril assembly can occur after secretion. Importantly, we show that blocking fibroblastic cell contraction inhibited the incorporation of endogenous purified LTBP-1 into a pre-existing FN network, indicating a cell-driven and active process. It remains to be shown whether fibroblasts use integrins to promote LTBP-1 fibril assembly or whether cell-derived strain generates assembly sites in FN for LTBP-1 binding, similar for what has been described for FN auto-fibrillogenesis (Singh et al., 2010b; Smith et al., 2007; Zhong et al., 1998). Experiments performed with pre-strained cell-free ECM render it unlikely that strain-induced FN auto-fibrillogenesis allows increased LTBP-1 binding and thereby contributes to increased TGF-β1 activation, at least in our culture experiments. In vivo however, this possibility has yet to be tested.

The fact that myofibroblasts formed focal adhesions with microcontact-printed LTBP-1 and that blocking of RGD binding integrins reduced adhesion of myofibroblasts to LTBP-1-coated substrates, suggests that fibroblastic cells directly bind LTBP-1. The RGD sequence described in human LTBP-1 serves as possible recognition site (Hyytiainen et al., 2004). However, rodent LTBP-1 and widely expressed LTBP-3 (Saharinen et al., 1999) do not contain RGD which may thus not be the only cell binding site in LTBP’s. Although direct integrin binding may contribute to LTBP-1 recruitment to existing FN fibrils, it seems not sufficient for LTBP-1 fibrillogenesis in the absence of FN.

It will be a future challenge to develop specific strategies interfering with the mechanical loading of the fibroblast/myofibroblast ECM with respect to TGF-β1 activation.
It is amply clear that defects in essential components of the FN and microfibril ECM lead to a variety of TGF-β1-related diseases (Baldwin et al., 2013; Doyle et al., 2012; Ramirez and Rifkin, 2009). The sole inhibition of LTBP’s binding to the ECM is unlikely to be efficient as an anti-fibrosis therapy since fibrillin-1 mutants defective for LTBP-1 binding exhibit TGF-β1 hyper-active phenotypes. Alternatively, integrins represent possible anti-fibrotic targets that provide relative cell- and tissue-specificity in the context of latent TGF-β1 activation (Gerber et al., 2013; Henderson et al., 2013; Hinz, 2013).

3.6 Appendix Chapter 3

3.6.1 Fibril quantification macro

The macro was written for ImageJ software and applied to quantify fibril formation.

```java
run("8-bit");
setAutoThreshold("Default dark");
//run("Threshold...");
setThreshold(X, 255);
run("Convert to Mask");
run("Analyze Particles...", "size=2-Infinity circularity=0.00-1.00 show=Nothing summarize");
X was determined for each experimental condition.
```
Chapter 4
The ED-A Domain in Fibronectin Enhances Binding of Latent TGF-β1 Binding Protein-1 to Fibronectin in the Fibroblast Matrix

The contents of this chapter are ready for submission as:

“The ED-A Domain in Fibronectin Enhances Binding of Latent TGF-β1 Binding Protein-1 to Fibronectin in the Fibroblast Matrix”

(to be submitted to Molecular Biology of the Cell)

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Chapter 4 continues on the findings of Chapter 3 that FN is essential for LTBP-1 incorporation and organization in the ECM. Here, I will focus on the question whether the myofibroblast characteristic FN splice variants ED-A plays a specific role in integrating LTBP-1 into the ECM. To test whether the presence of ED-A FN in the ECM enhances LTBP-1 incorporation in objective 3, I purified FN constructs and LTBP-1 from HEK293 conditioned medium. All experiments were performed by me, except for the pull-down experiments and confirmation of constructs, which have been carried out by Ms. Grace Chau.
4.1 Abstract

The excessive accumulation and contraction of collagenous ECM by myofibroblasts characterises fibrosis, a detrimental disease that causes organ failure with no effective therapy available to date. Myofibroblast activation from different precursor cells depends on mechanical stress, the presence of latent TGF-β1 in the ECM, and the ED-A splice variant of FN. However, the interplay between these factors is yet unknown. We hypothesize that ED-A FN plays a major role in regulating the bio-availability of TGF-β1 by promoting efficient binding of the TGF-β1 storage protein LTBP-1. Using culture substrates with defined elastic modulus, we show that increasing ECM stiffness results in increased expression and co-localization of LTBP-1 and ED-A FN in the ECM of human dermal fibroblasts. Competitive inhibition of the ED-A FN reduced the ability of fibroblasts to incorporate LTBP-1 in the ECM and lead to LTBP-1 accumulation in the cell culture supernatant. LTBP-1 exhibited reduced binding to full-length FN constructs lacking the ED-A domain than to ED-A FN in protein binding assays. Similarly, ED-A recombinant domain peptides bound LTBP-1 more efficiently than FN domain peptides lacking ED-A. Using dermal fibroblasts expressing specific FN splice variants confirmed that LTBP-1 preferentially binds to ED-A FN than other FN variants. Collectively, our results identify the ED-A domain as potential target for blocking TGF-β1 association with the ECM via LTBP-1. This knowledge will have a profound impact on future therapies to treat fibro-proliferative disorders.
4.2 Introduction

Wound closure is essential for proper wound healing to occur. Although healing may seem a simple process, it is complex and dynamic involving the interplay between a variety of different cells of which myofibroblasts play a central role. Myofibroblasts are characterized by expression of α-SMA that renders them highly contractile and efficient to close wounds by mediating ECM contraction (Hinz et al., 2001a; Tomasek et al., 2002). Myofibroblasts are responsible for ECM deposition and organization into a mechanically resistant scar. While ECM deposition during wound healing is beneficial, excess production and contraction of fibrous connective tissue is detrimental and defined as fibrosis. Abnormal stiffening and contraction of connective tissue in conditions of fibrosis is primarily responsible for a wide range of organ failures, including lung (e.g., pulmonary fibrosis), heart (e.g. after myocardial infarct), liver (e.g., liver cirrhosis), and skin (e.g., hypertrophic scarring and scleroderma) (Hinz et al., 2007). There is presently no effective therapy available for the treatment of fibrosis. Three elements are essential for myofibroblast differentiation: mechanical stress, active TGF-β1, and ED-A FN (Desmouliere et al., 2005; Gabbiani, 2003; Serini and Gabbiani, 1999). The absence of any of these three factors compromises myofibroblast differentiation and proper wound healing to occur. However, it is unclear how TGF-β1, ED-A FN and mechanical stress collaborate to promote myofibroblast differentiation. Recent findings using cultured human dermal, human cardiac, and rat lung fibroblast showed that latent TGF-β1 is activated from its storage site in the ECM by a process that requires cell contraction and a sufficiently stiff ECM (Klingberg et al., 2013; Sarrazy et al., 2014; Wipff et al., 2007).
TGF-β1 is non-covalently bound to the LAP forming the SLC and rendering the growth factor inactive (Robertson and Rifkin, 2013). SLC and LTBP-1 together form the LLC that is secreted by cells and acts as the main storage site for TGF-β1 in the ECM (Hyytiainen et al., 2004). The LLC interacts with two major structural ECM proteins, FN and fibrillin-1 (Hynes, 2009; Ramirez and Rifkin, 2009; Zilberberg et al., 2012a). Two major forms of FN are generated by alternative splicing from a single gene (Schwarzbauer et al., 1983): 1) soluble plasma FN (pFN) is secreted by hepatocytes into the circulation and 2) insoluble cellular FN (cFN) is secreted by a variety of different cells, including fibroblasts (Lahav and Hynes, 1981; Mosher, 1980; Ruoslahti et al., 1981). In contrast to pFN, cFN contains the ED-A and/or ED-B domains (Xia and Culp, 1995) (Figure 3).

In fibroblastic cells, the ED-A domain has been shown to be crucial for myofibroblast induction by TGF-β1 (Serini et al., 1998b). Conversely, TGF-β1 increases the expression of the ED-A FN isoform in fibroblast cultures (Balza et al., 1988). In the presence of the ED-A FN specific blocking antibody IST-9, and competitive recombinant ED-A peptide, rat lung fibroblasts were shown to exhibit significantly reduced levels of α-SMA positive myofibroblasts, leading to a decreased ability of these cells to contract 3D collagen lattices (Hinz et al., 2001a). These results are consistent with subsequent studies demonstrating abnormal wound healing of skin wounds of in EDA(−/-) mice (Muro et al., 2003). Another study by the same group showed that the ED-A domain in FN is essential for the resolution of lung injury through TGF-β1 activation (Muro et al., 2008). Collectively, these findings led us to hypothesise that ED-A FN is an essential factor in myofibroblast differentiation by controlling the storage of latent TGF-β1 in the ECM.
We tested this hypothesis by (1) studying colocalization of ED-A FN with LTBP-1 on differently stiff substrates, (2) testing if the binding of LTBP-1 to FN is dependent on the ED-A domain in - enzyme linked immunosorbent assay ELISA assays and (3) testing whether LTBP-1 is incorporated into the fibroblast ECM in the absence of the ED-A domain. Our results show that the ED-A domain in FN provides an important binding site for the LTBP-1 and thus controls TGF-β1 bio-availability for fibroblastic cells. Interfering with the ED-A FN-LTBP-1 interaction is a possible new strategy to treat fibro-proliferative disorders and organ failures, such as fibrosis.

4.3 Materials and Methods

4.3.1 Cell culture and reagents

Fibroblasts were explanted from human dermal tissue derived. In brief, tissues were cut into 1 mm$^3$ cubes, attached to tissue culture plates, and immersed in standard Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies), supplemented with 10% fetal bovine serum (Sigma-Aldrich), and penicillin/streptomycin (Life Technologies). Cells were allowed to migrate out of tissues for 10d before the first passage. Lineage lung fibroblast (MRC-5) cells were maintained in standard culture medium. HEK cells, stably expressing LTBP-1-EGFP (HEK-LTBP-1-EGFP) were maintained in Zeocin™ (1:1000, Life Technologies, Cat#R250-05) and G418 (1:50, Life Technologies, Cat#10131-035) according to Klingberg et al, 2013 (Klingberg et al., 2013).
HEK293 cells were transfected and stably selected with G418 (1:50, Life Technologies, Cat#10131-035) for expression of FN full length constructs. Small-interfering RNA constructs were designed against human FN and purchased from Thermo Scientific.

4.3.2 Purification of LTBP-1-EGFP, full length FN constructs and FN peptide domains

FN domain peptides were expressed and purified from *E. coli* according to (Kohan et al., 2010). LTBP-1 and full length FN His6-tagged constructs, were purified from serum-free conditioned media of transfected HEK293 cells. In brief, conditioned medium was collected and dialyzed against DPBS (Life Technologies) before it was run over an ion metal affinity chromatography column (IMAC) with HIS-Select® Nickel Affinity Gel (Sigma Aldrich, Cat#P6611). Columns were washed with wash buffer containing 0, 10, or 15 mM imidazole. Fractions containing LTBP-1 or full length FN constructs were eluted with 250 mM imidazole.

4.3.3 Immunofluorescence, microscopy, and quantitative image analysis

Samples were fixed with 3% paraformaldehyde for 10 min, washed with PBS, and then permeabilized with 0.2% Triton X-100 for 5 min. Primary antibodies used in this study were: α-SMA (mouse IgG2a, clone SM1, a kind gift of Giulio Gabbiani, University of Geneva, Switzerland), FN (1:400, Sigma-Aldrich, Cat#F3648), ED-A FN (1:200, Santa Cruz Biotech, Cat#sc-59826), LTBP-1 (1:100, R&D Systems, Cat#MAB388 or 1:250, Ab39, gift from Carl-Hendrik Heldin (Uppsala University, Sweden), GFP (1:2000, Abcam,
Secondary antibodies used were: goat anti-mouse IgG Alexa Fluor 568 (1:100, Life Technologies, Cat#A-11004), goat anti-mouse IgG1 FITC (1:100, Southern Biotechnology, Cat#1070-02), goat anti-mouse IgG2a TRITC (1:100, Southern Biotechnology, Cat#1080-03) and goat anti-rabbit-TRITC and -FITC (1:100, Sigma-Aldrich, Cat#F9887).

To stain filamentous actin and nuclear DNA, phalloidin Alexa Fluor 488 and 568 (1:100, Life Technologies, Cat#A-12379 and A-22287) and 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (1:50, Sigma-Aldrich, Cat#D9542) were used, respectively. Fluorescence images were acquired with a 40x or 60x objective mounted on an inverted microscope (Axiovert 135M; Carl Zeiss) equipped with a digital camera (C10600 Orca-R2; Hamamatsu). Confocal images were acquired at the Centre for Microfluidics Systems, University of Toronto, on a Nikon Eclipse Ti microscope system (Apo 60x) and SEM images were acquired on a Phillips XL30 ESEM at the Department of Pathology and Laboratory Medicine, Mount Sinai Hospital. Quantitative image analysis was performed using ImageJ (U. S. National Institutes of Health (NIH), Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2013) using customized macros (Hinz et al., 2007). Figures were assembled with Adobe Photoshop CS5 (Adobe Systems).

4.3.4 Western blotting

For Western blots, α-SMA, and vimentin were separated on 10% and FN, ED-A FN and LTBP-1 (non-reduced) on 8% SDS-Page gels. Gels were transferred to nitrocellulose membranes using semi-dry transfer technique at 18 mAmps/gel, 20 V, for 16 hrs or overnight. Protein membranes were blocked with 5% skim milk and primary antibodies...
were detected with fluorescently labeled anti-mouse-680 nm and anti-rabbit-800 nm IRDye® secondary antibodies (1:10000, LI-COR Biosciences, Cat#LIC-926-68020 and LIC-926-32211). Signals were detected with a LiCor Fx imaging system (LI-COR Biosciences).

4.3.5 ELISA

In order to study protein-protein interactions, we used a modified ELISA assay with fluorescent detection system. In brief, black clear bottom 96-well plates were coated overnight at 4°C with 10 µg of full length or domain peptides constructs of FN. Wells were then blocked with PBS and 0.5% BSA for 1h. After 3x washes with PBS, 10 µg of LTBP-1 was added to each well for 2h at 4°C. Afterwards, all wells were washed 3x with PBS and stained for LTBP-1 with anti-LTBP-1 and fluorescently tagged antibodies. LTBP-1 signal was detected in a LiCor Fx imaging system (LI-COR Biosciences).

4.3.6 Image analysis

ImageJ (NIH) was used to determine LTBP-1 fibril count and density (Klingberg et. al., 2014, in press). Briefly, single channel images were converted to 8 bit grey scale and thresholding was applied to remove background depending on experimental conditions. Particle events were counted and density analysed for events with a size larger than 2x2 pixels and 0-1 circularity.
4.3.7 Statistical analysis

When applicable, data are presented as means ± standard deviation (SD). We assessed differences between groups with an analysis of variance (ANOVA) followed by a post-hoc Tukey’s multiple comparison test and we set the significance level at p=0.05. For experiments comparing fibroblasts versus myofibroblasts, we performed a two-tailed paired t-test. When applicable, differences were considered to be statistically significant and indicated with *p≤0.05, **p≤0.01 and ***p≤0.005. Error bars represent standard deviation.

4.4 Results

4.4.1 ECM stiffness regulates expression of ED-A FN and LTBP-1

Previous studies identified mechanical stress as a major factor for myofibroblast differentiation (Serini and Gabbiani, 1999). However, the role of stress in regulating the expression levels of ED-A FN and LTBP-1 and their secretion into the myofibroblast ECM remains elusive. Therefore, we cultured primary hDf for 7d on compliant polydimethylsiloxane (PDMS) substrates with a Young’s modulus of 5, 100 or 3,000 kPa (Figure 21A). 5 kPa substrate were chosen to simulate the mechanical conditions of normal skin (Achterberg et al., 2014), 100 kPa substrates were used to simulate fibrotic tissue stiffness (Hinz, 2010b), and 3,000 kPa to provide mechanical growth conditions similar to conventional plastic culture (control). Immunofluorescence staining revealed that on 5 kPa ED-A FN is expressed to greater extent than LTBP-1. ED-A FN fibrils appeared short and thin but in large numbers. In the ECM of hDf grown on 100 kPa stiff
substrates, LTBP-1 co-localized with ED-A FN and formed longer fibrils compared to 5 kPa. At non-physiological high stiffness of 3,000 kPa, LTBP-1 fibrils were found that did not co-localize with ED-A FN (Figure 21A).

Figure 21: ECM stiffness affects α-SMA, ED-A FN and LTBP-1 expression.
Primary hDf were grown on compliant substrates with different stiffness, 3 kPa, 100 kPa and 3,000 kPa. Cells were immunolabelled for LTBP-1 (green) and ED-A FN (red). (B) Expression of ED-A FN, LTBP-1, α-SMA was determined by Western blotting. (C) Graph of WB quantification, normalized to vimentin as loading control, show mean values and standard deviations from at least three independent experiments (*p≤0.05, two-tailed paired Student’s t-test).
Figure 22: Co-IP of LTBP-1 and FN from hDf cultures.

hDMf were grown for 7d and lysates were used to immune-precipitate FN and LTBP-1 for detection of LTBP-1 and ED-A FN, respectively. (A) Control cell ECM (ECM) and supernatant (SUP), (B) cell ECM and supernatant (SUP) immune-precipitated with beads coated with anti-FN antibody (IP: FN) and (C) cell ECM (ECM) and supernatant (SUP) immune-precipitated with beads coated with anti-LTBP-1 antibody (IP: LTBP-1) were immuno-blotted for ED-A FN and LTBP-1.

Western blot analysis showed that ED-A FN and LTBP-1 were expressed on substrates with stiffness, 5, 100, and 3,000 kPa (Figure 21B). ED-A FN levels did not differ on 5 kPa versus 100 kPa but expression was markedly increased on 3,000 kPa (Figure 21C). The expression of the myofibroblast marker α-SMA increased with increasing mechanical stress (Figure 21C).

4.4.2 Co-immunoprecipitation (Co-IP) from human dermal fibroblast cultures

To test whether co-localization of LTBP-1 and ED-A FN was due to direct interaction between both proteins, we next cultured hDfs for 7d on conventional plastic culture dishes and collected cell lysates including the ECM for protein pull down assays (Figure 22).
Figure 23: Purification of full length FN constructs and FN domain peptides and ELISA.

(A) Schematic drawing of full length FN constructs FnA, FnB, FnAB and Fn0 with all domains displayed including the C-terminal 6xHis-tag. (B) Schematic drawing of FN peptides constructs, 11th, 11th-12th, 11-ED-A-12 and ED-A including the C-terminal 6xHis-tag. (C) Western blot of purified full length FN constructs stained for total FN, 6xHis-tag (rabbit), 6xHis-tag (m) and ED-A FN. (D) Purified FN peptides on Coomassie-blue stained, 6 % poly-acrylamide gel. (E) ELISA with full length FN constructs immobilized on TCP with LTBP-1 binding interaction measured. (F) ELISA with FN peptides immobilized on TCP with LTBP-1 binding interaction measured. Graphs of ELISA quantification show mean values and standard deviations from at least three independent experiments (*p≤0.05, two-tailed paired Student’s t-test).
Western blotting of full conditioned cell culture medium (SUP) and cell lysate (ECM) showed that the majority of LTBP-1 and ED-A FN protein was bound to the ECM (Figure 22A). In protein fractions from anti-FN coated beads, we detected a strong signal for LTBP-1 in the ECM and medium fractions, similar to the signal obtained from whole cell lysates. When beads were coated with anti-LTBP-1 antibodies and incubated with cell lysates, we were only able to detect ED-A FN in the ECM fraction but not in the cell supernatants. The fact that ED-A FN was not detected in the medium is indicative of its strong interaction with the ECM in an insoluble form (Figure 22C).

4.4.3 LTBP-1 binds specifically to the ED-A domain in FN

Having demonstrated in protein pull-down Co-IP experiments that LTBP-1 binds to ED-A FN in the ECM of hDfs, we overexpressed recombinant constructs of FN domains in *E. coli* and human embryonic kidney-293 cells (HEK293) (Figure 23A). Full length FN was produced in HEK293 in four different variants: FnAB containing the ED-A and ED-B domains; FnA containing ED-A but missing ED-B; FnB containing ED-B and lacking ED-A and Fn0 lacking ED-A and ED-B domain (Figure 23A). Furthermore, we purified short peptide fragments of FN domains from *E. coli* recombinant expression: 11th domain, 11th and 12th domain, ED-A domain and 11-ED-A-12 domain (Figure 23B). All proteins were purified using IMAC). Presence of purified proteins was confirmed on WB level for full length construct positive for 6xHis and cFN with ED-A positive bands for FnA and FnAB only (Figure 23C).
Figure 24: Blocking LTBP-1 interaction with FN in fibroblast culture.

(A) hDf cultures were grown for 7 days with the addition of FN domain peptides 11th, 11th-12th, 11-ED-A-12 and ED-A to the culture medium with daily replacement. Top row is stained for ED-A FN (red) and LTBP-1 (green) and bottom row only LTBP-1 channel (green) displayed. (B) WB of ECM after blocking for 7 days with staining for LTBP-1 and vimentin as loading control. (C) WB of supernatant after blocking for 7 days with staining for LTBP-1. Graphs in (B) and (C) represent WB quantification, normalized to vimentin as loading control for ECM, show mean values and standard deviations from at least three independent experiments (*p≤0.05, two-tailed paired Student’s t-test).
High yields of protein peptides were obtained and visualized in Coomassie blue stain (Figure 23D). We immobilized purified full length FN constructs in ELISA plates and subsequently added LTBP-1 (Figure 23E). LTBP-1 levels were measured and showed that LTBP-1 binds with two-fold signal increase to all full length constructs. FnA bound moderately more LTBP-1 than FnB and FnAB. In contrast, LTBP-1 signal increased to 8-fold when it was incubated with FnAB and purified LTBP-1 (Figure 23E). Incubation of LTBP-1 with FN domain peptides showed that LTBP-1 binds with significantly higher affinity to ED-A, 11-ED-A-12 and LTBP-1 (Figure 23F).

4.4.4 ED-A domain peptides competitively block LTBP-1 incorporation into hDf ECM

Due to the specific binding of the ED-A domain to LTBP-1 in ELISA, we next investigated whether competitive blocking of LTBP-1 incorporation into the ECM of hDfs is possible. HDfs were grown for 7d and FN domain peptides were added daily fresh to the culture at a concentration of 10 µg/ml. There was no interference of the FN domain peptides with the organization and incorporation of endogenous ED-A FN into the ECM (Figure 24A, upper row). However, endogenous LTBP-1 incorporation was reduced in the presence of the domain peptides, ED-A and 11-ED-A-12, respectively (Figure 24A, lower row). These results were confirmed by Western blot analysis of hDf ECM, showing 5-fold reduced signal for 11-ED-A-12 and 3-fold reduced signal for the ED-A domain compared to PBS-treated cultures (Figure 24B). LTBP-1 was released into the supernatant of hDfs and showed a decrease of LTBP-1 signal by 6-fold for 11-ED-A-12 and 3.5-fold for ED-A domain peptides compared to PBS-treated cultures (Figure 24B).
Figure 25: ED-A domain rescues LTBP-1 incorporation in fibroblast ECM.

(A) hDf were transfected with siRNA directed against FN. Western blotting analysis shows LTBP-1, FN, ED-A Fn and Fibrillin-1 expression 7 days after transfection. (C) Graph of FN siRNA knockdown efficiency from three independent experiments. Fold changes indicated reduction of fluorescent signal compared to control (B) Immunostaining of MRC-5 cells cultures after FN (siFN) and non-targeted knock-down (siNT) with labeling FN (red) and nuclei (blue). (D) MRC-5 cells transfected with FN full length constructs FnA, FnA, FnAB and Fn0 with FN (His6-tag, red), LTBP-1 (green) and nuclei (blue). (E) WB of full-length construct overexpression in MRC-5 cells. (F) WB and of down-regulation of endogenous FN and simultaneous over-expression of full length constructs in MRC-5. (G) LTBP-1 signal normalized to 6xHis-tag and vimentin signal.
4.4.5 Removal of the ED-A domain from full length FN reduces LTBP-1 incorporation into the ECM of hDf

In order to determine the role of the ED-A domain in cellular FN from fibroblast cultures, we transiently overexpressed the full length FN constructs (Figure 23A) into the human lung fibroblast line, MRC-5. However, MRC-5 expressed endogenous ED-A FN in their ECM. Therefore, endogenous cFN was down-regulated with specific siRNA directed against human FN (Figure 25A). Knock-down efficiency was 90% and resulted in co-down-regulation of LTBP-1 and fibrillin-1 (Figure 25B). FN fibrils disappeared in the ECM of fibroblasts (Figure 25C). Next, we transfected MRC-5 cells with full length FN constructs and confirmed FN presence via His-6 tag (Figure 25D). Overexpressed FN constructs incorporated and colocalized with endogenous FN (Figure 25D, E). Down-regulation of endogenous FN with simultaneous overexpression of full length FN constructs showed that 2-fold more LTBP-1 is incorporated into the ECM when ED-A domain was present (Figure 25F). Cellular FN overexpression resulted in 50% LTBP-1 incorporation when compared to control condition without down-regulation (Figure 25F).
4.5 Discussion Chapter 4

The role of the ED-A FN splice variant during myofibroblast differentiation and wound healing has been well established (Serini et al., 1998b; Serini and Gabbiani, 1999; White et al., 2008). Myofibroblast differentiation further depends on the presence of active TGF-β1 and mechanical stress arising from a stiff ECM. The interplay between these factors is suggested to determine the extent of fibrotic disease progression. However, it remains elusive how ED-A FN, TGF-β1, and mechanical stress interact to control myofibroblast differentiation. We hypothesized that the ED-A domain of FN sequesters TGF-β1 in the ECM, and that mechanical stress regulates expression levels and organization of ED-A FN and LTBP-1 in the ECM of myofibroblasts.

Our data demonstrate that ECM stiffness affects the presence of ED-A FN. In a previous publication from our lab, we showed that myofibroblasts need a sufficiently stiff ECM to release TGF-β1, possibly driving the production of ED-A FN in a feed forward fashion (Wipff et al., 2007). Consistently, the low mechanical stress produced by cells grown on soft culture substrates abrogated the expression of ED-A FN and LTBP-1 mimicking a healthy tissue state and an increased expression on pathological stiff substrates. The expression of ED-A and ED-B splice variant FN is up-regulated in fetal and tumor tissues, and during wound healing compared to low levels in normal adult tissues (Ffrench-Constant and Hynes, 1989) indicating the importance of tissue mechanics on the presence of the FN domains.
It has been shown that in a microenvironment with increased TGF-β1 levels provided e.g. by immune cells, fibroblasts favour the insertion of the ED-A domain within FN (Serini et al., 1998b). An ED-A FN specific integrin, α4β7, implicated in myofibroblast differentiation and attachment to ECM has been recently identified in murine lung fibroblasts (Kohan et al., 2010). During wound healing and in inflammatory skin diseases another α4-integrin, α4β1 is up-regulated in the presence of cytokines such as TGF-β1 and blocking antibodies against α4-integrins reduced the extent of bleomycin induced lung fibrosis in mice (Gailit et al., 1993; Wang et al., 2000). Both, integrins α9β1 and α4β1 recognize the EDGIHEL motif in ED-A FN which mediates cellular functions (Shinde et al., 2008) but have not been reported to bind latent TGF-β1 directly by recognizing RGD in LAP. The first integrin identified to bind to LAP and able to activate TGF-β1 was integrin αvβ6 expressed on epithelial cells (Munger et al., 1999).

However, for activation of TGF-β1 from the LLC by integrin αvβ6, binding of LTBP-1 to FN is required (Annes et al., 2004b). FN secretion and organisation into fibrillar structures precedes most other ECM components, highlighting its role as master-template for ECM assembly. Fibrillogenesis of LTBPs depends on FN (Dallas et al., 2005; Koli et al., 2005) and fibrillin-1 (Chaudhry et al., 2007; Isogai et al., 2003; Ono et al., 2009; Sabatier et al., 2009; Sabatier et al., 2013). Three different domains in LTBP-1, the first (hybrid), second or fourth 8-Cys domains, have been suggested to mediate association with the ECM (Unsold et al., 2001) (Figure 1). However, previous studies did not identify the domains in the FN molecule interacting with LTBP-1.
Our results further indicate that the ED-A domain in FN enhances the interaction with LTBP-1 with a potential influence on myofibroblast differentiation. We have indication that IST-9 antibody locking reduced binding of LTBP-1 to ED-A (data not shown) similar to blocking with ED-A domain peptide. However, presence of the of the 11th and 12th domain flanking ED-A in FN exhibited a greater blocking effect. Hence, we propose a model in which neighbouring domains support the binding of LTBP-1 to the ED-A domain in FN. Previous studies highlight the role of supporting domains such as heparin binding domains. In fact, LTBP-1 contains a sensitive proline-rich “hinge” region with a heparin binding consensus sequence (Chen et al., 2007). Chen and coworkers established a critical role of heparin sulfate proteoglycans (HSPGs) for controlling the storage of latent TGF-β1 in the ECM (Chen et al., 2007). FN further contains two heparin binding domains; one in proximity of the N-terminus and the other between the III12-14 domains (Clark et al., 2003). Adjacent domains such as the heparin binding domain neighbouring the ED-A domain in FN and unknown domains in LTBP-1 possibly enhance the interactions of both proteins. This fact is supported by recent literature showing that heparin-binding domains are able to regulate FN conformational changes and therewith availability of multiple growth factors in addition to TGF-β1, such as vascular endothelial growth factor (VEGF) (Hubbard et al., 2013; Mitsi et al., 2008).

In summary, we found that the mechanical conditions that fibroblasts encounter in advanced stages of wound healing and fibrosis influence ED-A FN and LTBP-1 expression and interaction. The ED-A domain seems to take a central stage in promoting FN interaction with LTBP-1 and stabilizing neighbouring domains in FN appear to enhance this binding.
We propose that blocking the interaction of LTBP-1 with the ED-A domain in FN is a potential strategy to reduce TGF-β1 storage in the myofibroblast ECM. Our model is supported by evidence from animal experiments showing that mouse lungs are protected from experimentally induced fibrosis by knocking-out the ED-A domain in FN (Muro et al., 2008).
Chapter 5 – Thesis Discussion and Future Directions

5.1 Discussion

Under normal conditions, fibroblasts maintain ECM homeostasis by keeping a fine-tuned balance between synthesis and degradation. In contrast, upon injury, fibroblasts become activated by the vast array of cytokines that are released during the immune response including the pro-fibrotic cytokine TGF-β1 and by the exposure to stress arising from the loss of ECM architecture (Tomasek et al., 2002). Under these conditions, fibroblasts differentiate into myofibroblasts expressing α-SMA and forming large focal adhesions at the end of actin-myosin containing stress fibers. (Darby et al., 1990; Goffin et al., 2006). Incorporation of α-SMA into stress fibers increases intracellular tension and enables the myofibroblasts to exert forces onto the ECM, thereby promoting ECM remodelling (Hinz et al., 2001a). The myofibroblast remodelling activity leads to stiffening of the surrounding ECM. In turn, myofibroblasts sense the stiff ECM and become activated in a loop that feeds myofibroblast persistence (Hinz, 2007; Hinz, 2012; Hinz et al., 2012). In addition to biophysical changes, the myofibroblast increases its expression of various ECM proteins, such as collagens and FNs (Vedrenne et al., 2012). ED-A FN has been shown to be essential for myofibroblast differentiation by TGF-β1 (Serini et al., 1998b). My thesis highlights the important interplay of TGF-β1, myofibroblasts and the ECM and sheds new light on the role of myofibroblasts in mechanically priming the TGF-β1 complex for subsequent activation. It defines a new role of the ED-A domain in FN in binding the LTBP-1.
5.1.1 The ECM as anti-fibrotic target

Given the clear role of the ECM as a mediator of fibrosis, it seems plausible that these proteins and their modifiers could be possible anti-fibrotic therapeutic targets. However, the ubiquitous nature and clear clinical importance of the ECM dictates that efforts need to be directed at identifying differences in ECM composition between normal and disease states. As an example, using mass spectrometry has evaluated differences in ECM composition between normal and fibrotic human lung, identifying a number of ECM molecules clearly overexpressed in the diseased organ (Booth et al., 2012b). Although further studies are needed to determine whether these changes reflect pathogenic mechanisms or are merely epiphenomena, we believe that ECM molecules, domains, or cross-links may offer possible novel therapeutic targets for patients with progressive fibrotic disorders.

Among the list of major myofibroblast ECM components, FN seems to be an appropriate target to control myofibroblast development and survival. However, the critical nature of FN to development indicates a need for specific targeting within the molecule. In this regard, the ED-A domain of FN is more attractive as a potential therapeutic target for the treatment of fibrotic diseases because it is a specific and crucial component of the myofibroblast ECM that is highly upregulated in a variety of fibrotic diseases but virtually absent from most normal connective tissues (Komblihrt et al., 1996; Muro et al., 2003; Muro et al., 2008). In addition to targeting ED-A FN, a second potential target currently under investigation is LOXL2 (Barry-Hamilton et al., 2010; Rodriguez et al., 2010).
Initial studies targeting LOX using the inhibitor β-aminopropionitrile (BAPN) reduced collagen crosslinking and scarring but did not proceed in clinical trials due to drug toxicity (Cox and Erler, 2011). However, LOXL2 has also been identified in, and is associated with, fibrotic tissues (Lugassy et al., 2012; Vadasz et al., 2005). LOXL2 antibodies are currently being considered for clinical trials in fibrotic disorders (Barry-Hamilton et al., 2010; Rodriguez et al., 2010).

5.1.2 Technological advances to measure stress-activated TGF-β1

TGF-β1 is stored within the ECM in a latent form which prevents binding to high affinity cell receptors. Latent TGF-β1 in combination with LAP are covalently linked to LTBP-1 mediating the binding to the ECM (Wipff and Hinz, 2008). Three principle mechanisms for TGF-β1 activation by cells have been reported (Jenkins, 2008; Sweetwyne and Murphy-Ullrich, 2012; Wipff and Hinz, 2008): 1) Binding to thrombospondin which induces a conformational change in LAP and releases TGF-β1 (Sweetwyne and Murphy-Ullrich, 2012). 2) Proteolysis of LAP which releases active TGF-β1 from the SLC and 3) integrin binding to a RGD sequence within the LAP protein and transmission of cell contraction forces that induce a conformational change in LAP and release active TGF-β1. In addition to the binding of integrins to LAP, RGD sequences have been identified in the LTBP moiety of the LLC (Sheppard, 2005; Wipff and Hinz, 2008). However, other techniques of TGF-β1 activation in a non-physiological and cell-independent fashion have been reported. Such treatments include heat (≥80°C), acidic or basic pH and different chaotrophic agents or detergents (Brown et al., 1990).
The assay that I developed in Chapter 2 is based on the principle of mechanical TGF-β1 activation. One major advantage of the assay is that different cell types can be used to produce the ECM. The cells are then removed using a mild detergent such as DOC which leaves behind the insoluble fraction of the ECM (Adams, 2002; Hedman et al., 1979). DOC treatment of FN ECM is routinely used to monitor the conversion of FN from the insoluble form into FN fibrils during assembly (Brenner et al., 2000; McKeown-Longo and Mosher, 1983).

The association of LTBP-1 with FN enabled us to use the DOC treatment as an efficient cell removal technique to produce an ECM loaded with TGF-β1 (Dallas et al., 2005; Koli et al., 2005). In Buscemi et al., we determined the force needed to open a single TGF-β1 complex molecule to be around 40 pN, which are in the range of forces of single integrins (Buscemi et al., 2011a). Various biophysical techniques have been developed for the spatial manipulation of micro-particles such as optical tweezers, micropipette aspiration and atomic force microscopy. (Applegate et al., 2004; Henriksen and Ipsen, 2004; Rico et al., 2005). The advantage that atomic force microscopy provides in detecting forces on the single molecule level was a major disadvantage in measuring the actual TGF-β1 molecule after release from the complex. The reason is that a single TGF-β1 molecule is below the detection limit of any TGF-β1 assay currently available. Two major TGF-β1 measurement assays are currently established for cells and tissues; 1) an ELISA and 2) the plasminogen activator inhibitor (PAI)-1 promoter/luciferase bioassay using TMLC with the latter being more sensitive (Khan et al., 2012).
Both assays require sufficient amounts of TGF-β1 and can only detect a global TGF-β1 release in the range of pM. Therefore, I employed the micro-bead pulling on a decellularized ECM and measured TGF-β1 in the media. However, microbead pulling experiments deliver 10-100-fold lower integrin forces suggesting that the threshold force for opening of the TGF-β1 complex cannot be reached (Litvinov et al., 2002; Weisel et al., 2003). The fact that we could still detect active TGF-β1 release argues for an average force over the number of possible integrin-ligand interactions on the bead surface.

I conclude that the average magnetic pulling force is sufficient to release TGF-β1 from the complex stimulating the TMLC reporter cells. However, not all integrins on the bead surface will be bound leading to an underrepresented TGF-β1 value. Importantly, magnetic microbeads can exert the forces that are needed to directly activate and release TGF-β1 from a LLC ECM in our experiments, demonstrating for the first time mechanical TGF-β1 activation in a cell-free system.

5.1.3 ECM strain controls the bio-availability of TGF-β1

The ECM of cells is well known for its function as stabilizer of the cells. It is a collection of molecules that are secreted and remodeled by cells providing structural support. The complex network of molecules such as proteins, glycoproteins and proteoglycans has evolved to be more than just an inactive, space-filing entity (Karsdal et al., 2013). We now know that the ECM composition differs between tissues and provides the environment for many different cell types which in turn use the ECM for adhesion, cell-to-cell communication and as a storage place for bioactive molecules.
In Chapter 3, I propose a novel mechanism in which cells mechanically prime the ECM for TGF-β1 activation by remodelling the ECM into fibrillar structures. To demonstrate *in vivo* relevance of my proposed mechanism, I have included a rat model comparing normal and mechanically strained full thickness wounds. The increased mechanical strain in the ECM during wound healing results in structural changes in latent TGF-β1-binding LTBP-1 that correlate with increased myofibroblast differentiation and the previously published upregulation of active TGF-β1 (phospho-Smad) signalling (Wipff et al., 2007). Using the same animal model, we have previously reported that tissue tension produced by fibroblastic cells in the granulation tissue increases accordingly over with time and with externally applied strain (Hinz et al., 2001b).

To decipher the mechanistic basis for these *in vivo* observations, I stimulated hDfs with TGF-β1 and demonstrated upregulation of LTBP-1 expression. This result is in accordance with the reciprocal positive forward loop of myofibroblast differentiation leading to ECM changes promoting fibrosis (Blaauboer et al., 2014). Interestingly, TGF-β1 stimulated the secretion of LTBP-1 which stores the TGF-β1 in the ECM. Closing the loop, LTBP1s can in turn direct the TGF-β1 action at multiple levels, such as folding and secretion of the molecule, temporal and spatial deposition in the ECM and regulation of activation (Todorovic and Rifkin, 2012).

Previously, the temporal and spatial deposition of LTBP-1 was thought to be dependent on a pre-existing FN ECM because FN is one of the initial proteins secreted by fibroblasts (Dallas et al., 2006). In cultured human lung fibroblast, LTBP-1 colocalized with FN as early as 2d (Koli et al., 2005).
I could confirm an association of LTBP-1 and FN but overexpression studies with our GFP-labeled construct revealed that LTBP-1 is secreted independently in an unorganized “dot-like” fashion. Over time LTBP-1 and FN colocalization and fibril formation increases. Furthermore, I could show that LTBP-1 organization into fibrils enhances the ability of cells to release TGF-β1 and early work from Flaumenhaft et. al. suggested that LTBPs concentrate the latent TGF-β1 on the surface of the cells (Flaumenhaft et al., 1993). Because protein concentration increased in fibrils (higher fibril density), I conclude that enhanced fibrillogenesis of LTBP-1 impacts the ability of cells to access and activate the TGF-β1.

The myofibroblast has been described as a mechanically active cell with respect to ECM remodelling (Hinz, 2010b). In my research, I could show that myofibroblasts have a higher capacity to remodel a pre-produced ECM into fibrils than fibroblast. A possible reason is that myofibroblast form larger focal adhesions, contact points with the ECM, enabling to transmit higher intracellular forces to ECM molecules such as FN (Hinz et al., 2003; Hinz and Gabbiani, 2003). Myofibroblast contraction organized the “dot-like” structures into fibrils. This contraction occurs on a low-amplitude and frequency level and can result in macroscopic contractures of ~1 cm per month in tissues (Castella et al., 2010). This contraction may result in significant tissue contractions over days, weeks and months (Tomasek et al., 2002). My novel work highlights this process for LTBP-1 fibril formation on a single cell basis and adds a physiological relevance of the applied force.

The results obtained with splinted rat wounds demonstrated that mechanical loading of ECM and tissues can alter gene expression. Mechanical loading is defined as the
imposition of stresses or strains through application of physical forces (Wang et al., 2007). However, I was interested in whether externally applied strain to a pre-laid down ECM can alter TGF-β1 release. Therefore, I applied equi-biaxial strain to compliant substrate and was able to stretch LTBP-1 positive ECM fibrils. This method does not fully mimic the *in vivo* situation as cells are embedded in a 3D environment (Eastwood et al., 1998). An interesting study used fibroblasts embedded within a collagen ECM with defined microstructure (Pizzo et al., 2005). Three-dimensional strain changes and the distribution of β1 integrin were quantified. Interestingly, fibroblasts in a low-fibril density environment organized the ECM to greater extent and β1 integrin localization was related to local strain (Pizzo et al., 2005). These findings are consistent with my results that fibroblast organize their ECM to increase fibril density and highlight the role of β1 integrin in this process, my suspected candidate for LTBP-1 binding. Cellular contraction transmitted to an ECM fibre leads to the extension of the fibre. In my own strain studies, I followed published values of a maximal FN fibre extension of up to 3-fold by external means (Little et al., 2008; Ohashi et al., 1999). Extension of FN fibrils by strain was shown to reveal hidden cryptic binding sites (Klotzsch et al., 2009). It is conceivable that a similar mechanism applies to LTBP-1 which has yet to be studied. Precondition of such a mechanism to work is direct exertion of force to the LTBP-1 molecule.

5.1.4 The role of integrins and cell-ECM adhesions in organizing LTBP-1

In addition to regulating ECM protein expression, TGF-β1 increases the expression of several integrins such as αvβ3, αvβ5, αvβ6, and several β1 integrins (Heino et al., 1989; Heino and Massague, 1989; Ignotz et al., 1989; Sheppard et al., 1992; Zambruno
et al., 1995). Upregulation of these integrins will contribute to the feed-forward loop of TGF-β1 and myofibroblast auto-activation because the same integrins αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, and α8β1 have been shown to promote TGF-β1 activation by binding to the RGD-site in LAP (Annes et al., 2002; Araya et al., 2006; Lu et al., 2002; Ludbrook et al., 2003; Mu et al., 2002; Munger et al., 1998). However, studies investigating the binding of integrins to LTBP-1 are missing and it remains elusive if integrins directly bind to LTBP-1 or if LTBP-1 needs to be bound to a “carrier” protein such as FN or fibrillin-1. I was able to use the purified LTBP-1 in μCP and integrin blocking experiments. My new data now show that blocking RGD-recognizing integrins inhibits adhesion and spreading of myofibroblasts to LTBP-1-coated culture substrates. RGD binding turns out to be essential. In light of the new data, integrin β1 is the strongest candidate for LTBP-1 binding through RGD but other αv integrins appear to be able to promote cell adhesion to LTBP-1 to different extents. It still remains a challenge and a lot more work needs to be done in order to identify which α and β1 integrin subunit combination is binding to LTBP-1, as there are 11 possible combinations (Hynes, 2002). Despite, μCP and integrin blocking experiments have been performed in serum-free media and with purified LTBP-1, a small chance of FN contamination is possible.

Hence, FN assembly and deposition is an α5β1 integrin–dependent process, we cannot fully exclude that β1 integrin adhesion may be via cell produced FN that interacts with LTBP-1 (Singh et al., 2010b). Nevertheless, β1 integrin is a good candidate for our force mediated LTBP-1 organization and priming concept because integrin α5β1 can switch between a relaxed and tension state in response to myosin II-generated cytoskeletal force (Friedland et al., 2009).
From my adhesion studies with the purified LTBP-1, I developed that LTBP-1 fibril formation is a focal adhesion-dependent process. Focal adhesions are the contact points of the cell with its surroundings and link inside signals to the outside and vice versa (Geiger et al., 2001). A key molecule in focal adhesions is FAK that is rapidly phosphorylated in response to interactions with FN (Hanks et al., 1992). Moreover, FAK is responsive to TGF-β1 and induces ECM contraction possibly effecting FN assembly (Liu et al., 2007). In my research, I used the FAK knock-out model to create an ECM that has a defect in FN organization as previously described (Illic et al., 2004). Using this model in combination with my developed micro-bead assay, I was able to compare differently structured ECM with equal TGF-β1 amount rather than noticing the difference in cellular knock-out phenotype. My novel results show that organization defects manifested during ECM remodelling in FAK-/- cells lead to reduced TGF-β1 release. TGF-β1 can induce myofibroblast differentiation by integrin signalling via FAK (Thannickal et al., 2003). And elevated levels of FAK correlate with high levels of TGF-β1 in cancer progression (Golubovskaya et al., 2009; Zhao and Guan, 2009). First clinical trials targeting FAK seem promising and results are encouraging (Golubovskaya, 2014).

Besides interfering with FAK as a general focal adhesion component, targeting specific myofibroblast integrins emerges as another promising therapeutic approach to treat fibrosis (Gullberg, 2009; Henderson and Sheppard, 2013). Many integrins contribute to fibrosis and myofibroblast differentiation through various pathways, including integrin α3β1 (Kim et al., 2009), glycated integrin α11β1 (Carracedo et al., 2010; Talior-Volodarsky et al., 2012), integrin αvβ3 (Hinz, 2006), integrin α4β7 (Kohan et al., 2010), and β1 integrin (Chan et al., 2010; Liu et al., 2010).
Of particular interest, αvβ6 integrins, necessary for epithelial activation of TGF-β1 (Annes et al., 2004b), and αvβ5 integrins, involved in mesenchymal cell activation of TGF-β1, have emerged as potential targets in fibrotic disorders. Currently, antibody therapy to αvβ6 integrins is being tested in a Phase 2 trial of patients with idiopathic pulmonary fibrosis (Clinicaltrials.gov identifier NCT01371305).

5.1.5 ED-A FN is potent anchor for latent TGF-β1 in the myofibroblast ECM

FN is one of the major binding partners of LTBP-1 but the direct interaction has not been associated with a disease model. In a LTBP-1 knock-out model, mice showed more compact head structure with shortened snouts cultured hepatic stellate cells failed to differentiate into myofibroblasts, possibly due to a lack in TGF-β1 bioavailability (Drews et al., 2008). In contrast, fibrillin-1, the second major LTBP-1 binding partner has been extensively studied in the disease Marfan syndrome. A mutation in fibrillin-1 leads to ineffective binding of the LLC and misregulated TGF-β1 activation (Neptune et al., 2003). Furthermore, the interaction of LTBP-1 and FN is essential for mechanical TGF-β1 activation by integrin αvβ6 (Annes et al., 2004b; Fontana et al., 2005). A recent study showed that in fibrillin-1 knock-out (Fbn1/-) fibroblast cultures, LTBP-1 is still deposited but when FN assembly is blocked LTBP-1 cannot be incorporated (Zilberberg et al., 2012a). One of my aims was to further investigate this finding by using a different approach. Labeled LTBP-1 was supplied in excess on coated substrates and FN knock-down hDfs or knock-out mouse fibroblasts were monitored for their ability to form LTBP-1 fibrils. The FN knock-down and knock-out had a severe effect on the fibroblasts ability to form fibrils.
Less fibrils were formed providing the new information that FN is necessary for LTBP-1 fibrillogenesis. In conclusion, the presence of FN enhances LTBP-1 fibril formation and may indicate another regulatory step. Blebbistatin is a non-muscle myosin II contraction inhibitor (Straight et al., 2003). Using blebbistatin, I was able to block the incorporation of LTBP-1 into a pre-existing ECM. This result provides further evidence for a contraction mediated LTBP-1 fibril assembly process.

In Chapter 4 of my thesis, I established a novel specific interaction between LTBP-1 and the ED-A splice variant of FN and provided first indications that this interaction is possibly regulated by mechanical stress. It has been well established that the stiffness of the ECM regulates myofibroblast differentiation and α-SMA expression (Hinz, 2009; Huang et al., 2012). I now complement these findings by showing that both LTBP-1 and ED-A FN incorporation into the myofibroblast ECM is upregulated with increasing ECM stiffness. My results are consistent with the expression profile of ED-A FN and LTBP-1 in vivo, being upregulated during fibrosis (Lepparanta et al., 2012; White et al., 2008). Interestingly, ED-A domain knock-out mice are viable, fertile but exhibit defective wound healing and are protected against pulmonary fibrosis (Muro et al., 2003; Muro et al., 2008).

In previous publications immunofluorescence double labeling of FN and LTBP-1 was performed and showed colocalization (Taipale et al., 1996). However, this study did not perform pull down experiments and did not look at FN domain specific binding of LTBP-1. My work expands this knowledge by adding pull down experiments with immobilized FN and LTBP-1 and vice versa.
I immobilized either FN or LTBP-1 and was able to immuno-precipitate the respective other. The current model of LTBP-1 association with the ECM suggests that the N-terminal region of LTBP-1 interacts first with the ECM followed by the C-terminal region (Unsold et al., 2001). However, both regions can independently bind to the ECM (Unsold et al., 2001). In vivo work truncating the ECM binding C- and N-terminal regions of LTBP-1 lead to improper ECM incorporation of the LLC and to the pre-mature activation of TGF-β1 (Mazzieri et al., 2005).

Only limited knowledge is available from current literature concerning the FN side the protein-protein interaction. One study blocked FN assembly with a N-terminal FN fragment reducing LTBP-1 in the ECM and in FN knock-out animals only full-length FN was able to rescue LTBP-1 incorporation (Dallas et al., 2005). To define the FN region/domain that mediates the binding to LTBP-1, I purified full-length FN constructs lacking either ED-A and/or ED-B domain and 11th, 11-12th, ED-A and 11-ED-A-12 peptide domain constructs. Immobilization of the FN constructs and probing for labeled LTBP-1 revealed that LTBP-1 preferably binds to constructs containing the ED-A domain. The addition of the domain peptides to a fibroblast culture over 7 days showed that the ED-A domain as a peptide can competitively block LTBP-1 association with the ECM.

To provide direct proof for ED-A FN-to-LTBP-1 binding, I down-regulated endogenous FN and reintroduced full length FN lacking the ED-A and/or ED-B domain. Cellular FN, containing both ED-A and ED-B domain was only partially able to rescue LTBP-1 incorporation. The lack of ED-A domain may be a reason why ED-A knock-out mice have a significantly shorter life span and wound-healing defects (Muro et al., 2003).
5.2 Overall Conclusion

In summary, my experimental data show that both the myofibroblast and its ECM are critical contributors to pathologic fibrogenesis in a variety of organs. Fibroblastic cells not only acutely control TGF-β1 activity via secretion or proteolysis but also by inducing long-lasting changes in LTBP-1 fibril organization. ECM fibril strain determines the threshold that cell contractility needs to overcome to release TGF-β1. In addition, the amount of LTBP-1 bound to the ECM is regulated by the expression of major ECM proteins such as FN or fibrillin and their respective splice forms and domain structures. The combination of ECM composition, e.g. amount of ED-A FN, and fibril structure contributes the TGF-β1 biology and availability. Our knowledge in this arena has provided the foundation for upcoming and current clinical trials in patients with fibrotic disorders. Further investigation into the mechanisms by which the ECM promotes fibrosis will likely identify other promising potential targets for therapeutic intervention.

5.3 Future Directions

Over the past years, many markers of fibrosis have been established and we now know that ECM proteins and their modifiers play a significant role in fibrosis prediction and progression. However, discovery of powerful anti-fibrotic drugs from potent target candidates has been insufficient. Despite the fact that clinical trials have been initiated with promising anti-fibrotic drug candidates, there is still a lack of specific biomarkers to measure fibrosis regression (Schuppan and Kim, 2013).
Moreover, the lack of specificity of drug candidates is the most common reason for failure in animal or clinical human trails. In most cases, drug candidates are very specific but fail to meet clinical success criteria. This is due to the fact that the development of fibrosis is not as a single-cause event. Different injury models, acute or chronic, can give rise to a fibrotic lesion and we need to focus our efforts on customized therapy options by pursuing multiple approaches in parallel.

This thesis highlights the ECM as a crucial point of contact for fibroblastic cells. Dysregulation of either cellular remodelling or ECM deposition can turn a tissue from normal into disease state. As many approaches aim at the suppression of the myofibroblast phenotype, the beneficial ECM synthetic activities of these cells is often essential in tissue repair. It is rather their persistence after wound resolution that marks the onset of fibrosis. Hence, eradicating the myofibroblast will create a void space in wound healing and will most likely lead to non-healing wounds such as ulcers (Mustoe, 2004). Not only has the structure of the ECM an impact on TGF-β1 activation but also the protein composition such as the amount of ED-A domain containing splice variant of FN.

In addition to my work, many studies have shown that various ECM molecules are overexpressed in diseased tissues (Booth et al., 2012a). And here again, we can ask the hen and egg question of whether the changes in myofibroblast phenotype drive ECM synthesis and remodelling or whether the changes in the ECM cause myofibroblast persistence and which of the two is major.
In addition to collagen, FN is one of the major ECM components present in myofibroblast ECM and is upregulated during fibrosis. Furthermore, FN growth-factor binding domains are necessary for fibroblast survival (Lin et al., 2011). Therefore, targeting FN in myofibroblast survival seems obvious. However, a general approach of FN reduction would be fatal as FN is required for assembly of many essential ECM components such as collagen and microfibrils. A more specific approach of targeting FN domains that are highly upregulated in fibrosis but absent in normal tissues is preferred. The ED-A domain in FN has been show to present such an expression pattern (Kornblihtt et al., 1996; Muro et al., 2003; Muro et al., 2008). My results show that the amount of LTBP-1 in the ECM of myofibroblast is dependent on the ED-A domain in FN. We therefore suggest focusing on ED-A domain blocking studies for fibrosis repression.

Key mediators between the inside of cells and the ECM are integrins. This group of cell membrane receptors is a promising target for fibrosis therapy (Gullberg, 2009). All αv-integrin receptors with RGD-site specificity have been reported to bind to the LAP of the small latent complex and may contribute to TGF-β1 release in one or other way. Of particular interest is the αvβ6 integrin, which is associated with epithelial activation through TGF-β1 (Horan et al., 2008). Currently, an antibody against αvβ6 integrin reached phase 2 in a clinical trial for idiopathic pulmonary fibrosis (Clinicaltrials.gov identifier NCT01371305). Our research utilized the high affinity αvβ6-integrin to LAP to measure TGF-β1 release from differently organized ECM. Finally, interfering with the mechanical state of the ECM provide a possible entry point to interfere with the feed-forward loop of fibrosis progression.
Remodelling of the ECM is a complex and dynamic process that is controlled by cells and can lead to different outcomes of wound healing (Cox and Erler, 2011). Structural changes can manifest in mechanical properties of the ECM favouring myofibroblast persistence. A stiff ECM provides a clue for myofibroblast differentiation divining the vicious loop of self-activation. In my thesis, I established the link between ECM organization and TGF-β1 activation. It sounds promising to develop drugs that will target that link and interfere with the cells ability to pre-stress the ECM. However, it will not be sufficient to block ECM remodelling at the level of fibril organization without targeting the myofibroblast. Therefore, a strategy based on the combination of reducing myofibroblast differentiation, cell to TGF-β1 complex binding and ECM remodelling will most likely be effective as it aims at different angles of fibrosis development and progression.
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