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Biochemical and biomechanical regulation of the myofibroblast phenotype

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CHAPTER
1

INTRODUCTION AND AIM OF THIS THESIS

CHAPTER 1

BACKGROUND

Most cells in the human body are supported by an intricate network of macromolecules termed extracellular matrix (ECM)[1]. An important function of the ECM is to provide structural support for cells during development and through adult life in homeostasis and disease. Each organ, or functional organ unit, is characterized by a unique ECM signature, specific for supporting the functions of the resident cells and microenvironment. Next to offering physical support, ECM guides segregation, establishment, and maintenance of cell differentiation status[2]. To achieve these ends, ECM can be found as interstitial arrangement of interconnected subunits, or as highly specialized frameworks such as the epithelial and endothelial basement membrane[2]. Additionally, ECM acts as a depot for growth factors, receptors, and hormones, and regulates tissue hydration and pH. This multi-functionality draws from the complex biochemical and biomechanical interactions between proteins, carbohydrates, and water, which create unique adhesion surfaces and form physical barriers between different cell layers. These interactions result in a tissue-specific composition and architecture that perfectly fits the functions of the inhabiting cells[3]. As the microenvironment in an organ changes, so does the ECM. Cell-ECM interactions are highly dynamic and usually involve the synthesis and higher order interconnection of new ECM macromolecules and enzyme-dependent post-translational modifications. Thus, ECM components can provide physical support for cells and simultaneously act as active modules in signaling to guide cell growth, migration, function and fate[2]. However, when the homeostasis of an organ is disturbed, aberrant regulation of ECM synthesis, remodeling and degradation may have disastrous effects on organ function. In this thesis, I set out to uncover yet unknown regulators of ECM-related disorders such as fibrosis and cancer. In this chapter I will give a brief introduction of the key components and functions of the ECM and illustrate how cells and ECM can interact to regulate cell function in health and disease.

KEY MOLECULES IN EXTRACELLULAR MATRIX

The most abundant components of the ECM are collagens; large rod-shaped proteins designed to give the ECM its mechanical and structural properties. The human genome encodes for 28 types of collagens and their structural hallmark are the triple helices that, depending on the combination of α-chains, can form either non-fibrillary structures or self-assemble into collagen fibrils[1] (**Figure 1**). The triple helix consists of α-chains with the repetitive amino acid sequence Gly-X-Y, where X and Y can be any amino acid. The X and Y positions are often occupied by hydroxylated forms of lysine and proline, amino acids mainly found in collagens[2]. Collagens also contain non-helical regions that can be short (in case of the N- and C-terminal telopeptides in the fibril forming collagens) or can cover the larger part of a collagen molecule (seen in large stretches of collagen XII)[4]. The main fibril-forming collagens (type I, II, III, V and XI) make up about 80% of the total collagen mass in the human body, and provide tensile strength in vessels, bone, tendon, skin, and cartilage[4]. Non-fibrillary collagens (such as collagen type IV, VIII and X) form networks, associate with fibril networks (collagen type

IX, XII and XIV), occur as transmembrane molecules (collagen type XIII and XVII), or form flexible microfibrils that assemble into filamentous networks (collagen type VI)[4]. Type IV collagen forms chicken-wire-like structures and is one of the main components of the epithelial and endothelial basement membrane. Cells are connected to collagens through integrin receptors and discoidin domain receptors (DDR), each recognizing specific amino acid residues that allow simultaneous binding and signaling[2,5]. These connections between cells and collagens are crucial for cell motility, proliferation, cytokine secretion and ECM remodeling[2,5]. Collagen biosynthesis encompasses a series of intra- and extracellular post-translational modifications performed by a variety of enzymes, collectively termed the 'collagenome'. During disease, changes or aberrations in collagen biosynthesis may lead to altered levels of collagen production. In turn, excessive levels of collagens may result in the alteration or even destruction of tissue architecture[4].

A second major component of the extracellular space is the family of fibronectins, massive glycoproteins that connect the structural components of the ECM to each other and to cells[6]. Many of the ECM components described above interact through connections with the multi-domain protein fibronectin. Fibronectins are secreted by the cell as large glycoproteins that assemble into fibrillary structures and connect the cell to its surrounding ECM via integrin receptors[7]. Fibril formation is mainly dependent on the α5β1 integrin and requires coordinated expression of both molecules. Each fibronectin subunit consists of three types of modules termed repeats, each of which has a distinct structure: type I, II and III repeats. These subunits are designed to specifically aid in fibronectin self-assembly or associate with other ECM proteins or receptors, including gelatins, fibrin, heparins, integrins, or membrane-bound syndecans[6]. Fibronectin assembly involves the interactions of a specific pair of type III repeats called the Arg-Gly-Asp (RGD) motifs with integrins on the cell membrane. Alternative splicing of the fibronectin transcript generates different mRNAs that in turn code for specific protein isoforms (e.g. cellular and plasma fibronectin), each with distinct functions. Cellular fibronectin is a multimeric insoluble protein that includes the type III repeats that form the so called EDA and EDB segments, and are thought to play a role in cell adhesion and fibril stability, respectively. Plasma fibronectin lacks the EDA and EDB segments and circulates in the bloodstream as dimeric soluble protein. What has become clear is that EDA and EDB modules are marginally expressed during normal organ homeostasis, but are increased during injury and disease[6].

Another group of molecules comprising ECM are the proteoglycans. Proteoglycans consist of a core protein that is heavily glycosylated through the covalent binding of one or more glycosaminoglycans (GAGs)[2] and can be classified into three major groups based on the type of GAG side chain. GAGs include heparin sulphate, chondroitin sulphate, dermatan sulphate, hyaluronan, and keratan sulphate. One of the primary functions of proteoglycans is to regulate the biochemical and hydrodynamic characteristics of ECM that can be assigned to their signature GAG repeats, which bind water and provide hydration and compression resistance to tissues[2]. Proteoglycans can also act as a depot for growth factors and hormones and are known to bind to
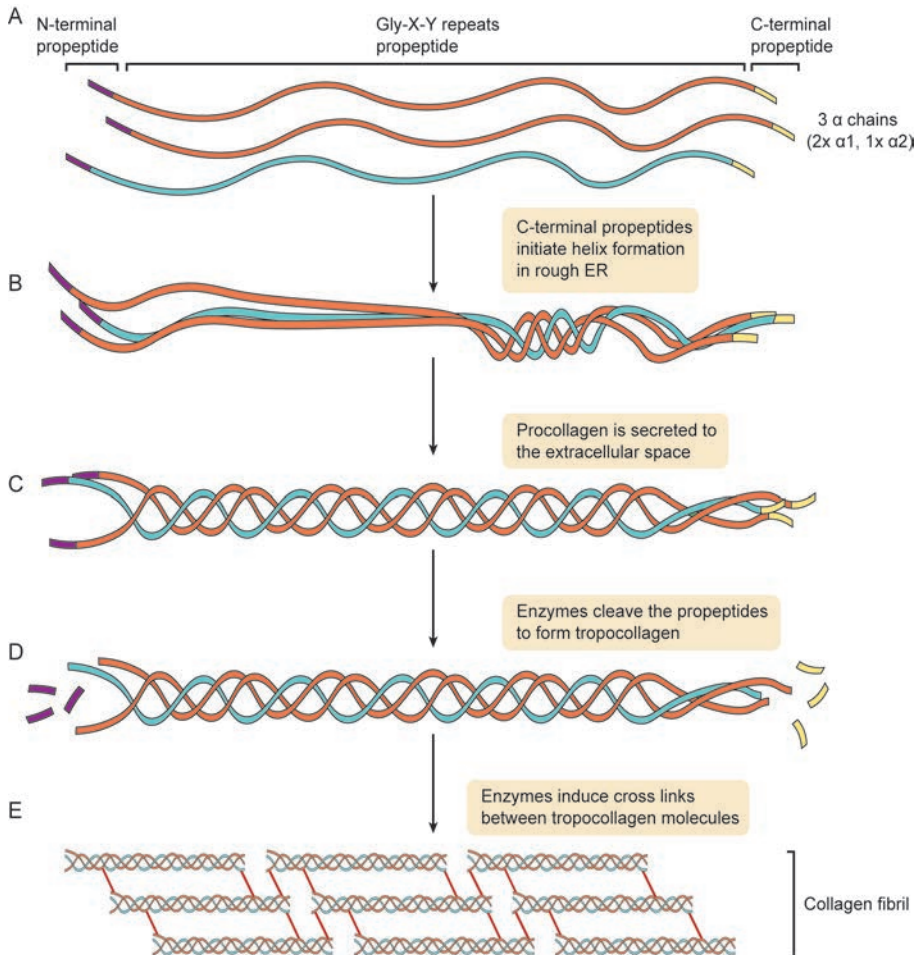
A N-terminal propeptide — Gly-X-Y repeats propeptide — C-terminal propeptide

3 α chains (2x α1, 1x α2)

C-terminal propeptides initiate helix formation in rough ER

B

Procollagen is secreted to the extracellular space

C

Enzymes cleave the propeptides to form tropocollagen

D

Enzymes induce cross links between tropocollagen molecules

E

Collagen fibril

Figure 1. Schematic representation of collagen biosynthesis.

a wide variety of ECM components, such as laminin and fibronectin[8]. In addition to being constituents of ECM, certain proteoglycans are known to be involved in the assembly of collagen fibrils, including the small leucine-rich repeat proteoglycans decorin and biglycan[9]. Taken together, the interconnection between different ECM modules creates a complex and tailored meshwork for cells to guide their function and fate. Additional post-translational modifications such as proteolytic cleavage, citrullination, cross-linking, nitrosylation, and glycosylation provide another tier of ECM specialization. Cells interact with the ECM through specialized receptors and membrane-bound protein complexes including integrins, DDRs, and cell surface proteoglycans. In this respect, cells transduce biochemical and biomechanical signals

to the ECM and vice versa, in order to maintain tissue homeostasis. A full description of ECM and its functions is beyond the scope of this thesis (for excellent review articles see[1,2,8]).

EXTRACELLULAR MATRIX IN HOMEOSTASIS AND DISEASE

All cell types synthesize and deposit ECM macromolecules required for their function, thus participating in maintaining tissue homeostasis. In response to tissue injury, the human body initiates mechanisms that drive immediate repair and remodeling of the injured site. The so-called wound healing response is characterized by three stages: inflammation, cell proliferation, and finally the maturation and remodeling of the newly synthesized ECM. Initially, these stages were used for the description of dermal wounding, but are readily applicable to the healing processes of multiple organ systems[10], with exception of the central nervous system. The timing and amplitude of wound healing are key for proper restoration of tissue morphology and function.

Chronic organ injury causes a disturbed and prolonged wound healing response, with aberrant deposition and remodeling of the newly synthesized ECM components—termed fibrosis (**Figure 2**). Organ fibrosis often starts with repeated injury of epithelial or endothelial cells, which release pro-inflammatory molecules, including cytokines and chemokines such as transforming growth factor (TGF)β1. Mononuclear inflammatory cells migrate toward the site of injury where they consume cellular debris, remove dead and dying cells, and produce even more cytokines. The secretion of ECM degrading enzymes promotes the activation of effector cells—such as pericytes and fibroblasts—and the enzymatic release of ECM bound cytokines[11]. Eventually, more effector cells migrate into the wound area and undergo a phenotypic shift toward myofibroblasts: cells specialized in ECM production, contraction and remodeling[12]. The balance between proteolytic degradation and synthesis shifts towards excessive ECM deposition and together with a disturbed remodeling, result in a haphazard accumulation of ECM components. Increased cross-linking of the collagen molecules by both enzymatic and non-enzymatic means make the collagen difficult to degrade and causes loss of tissue functionality and architecture, and at the same time results in stiffening of the ECM. In addition to distorting the normal tissue architecture, fibrous ECM alters the behavior and function of the cell types present, by means of both biochemical and biomechanical signaling, eventually leading to chronic organ dysfunction[10,13–15].

The key cells in fibrotic disorders are the so-called myofibroblasts, characterized by a synthetic and contractile phenotype. In the early phases of wound healing, local effector cells migrate into the injured site and acquire bundles of microfilaments that contain β- and γ-cytoplasmic actins[12]. Over time, through the action of growth factors such as TGFβ1, together with de novo expression of cellular fibronectin, myofibroblasts start to express the smooth muscle cell actin isoform smooth muscle alpha actin (SM α-actin or αSMA), which is thought to be essential for the contractile phenotype[12,16].
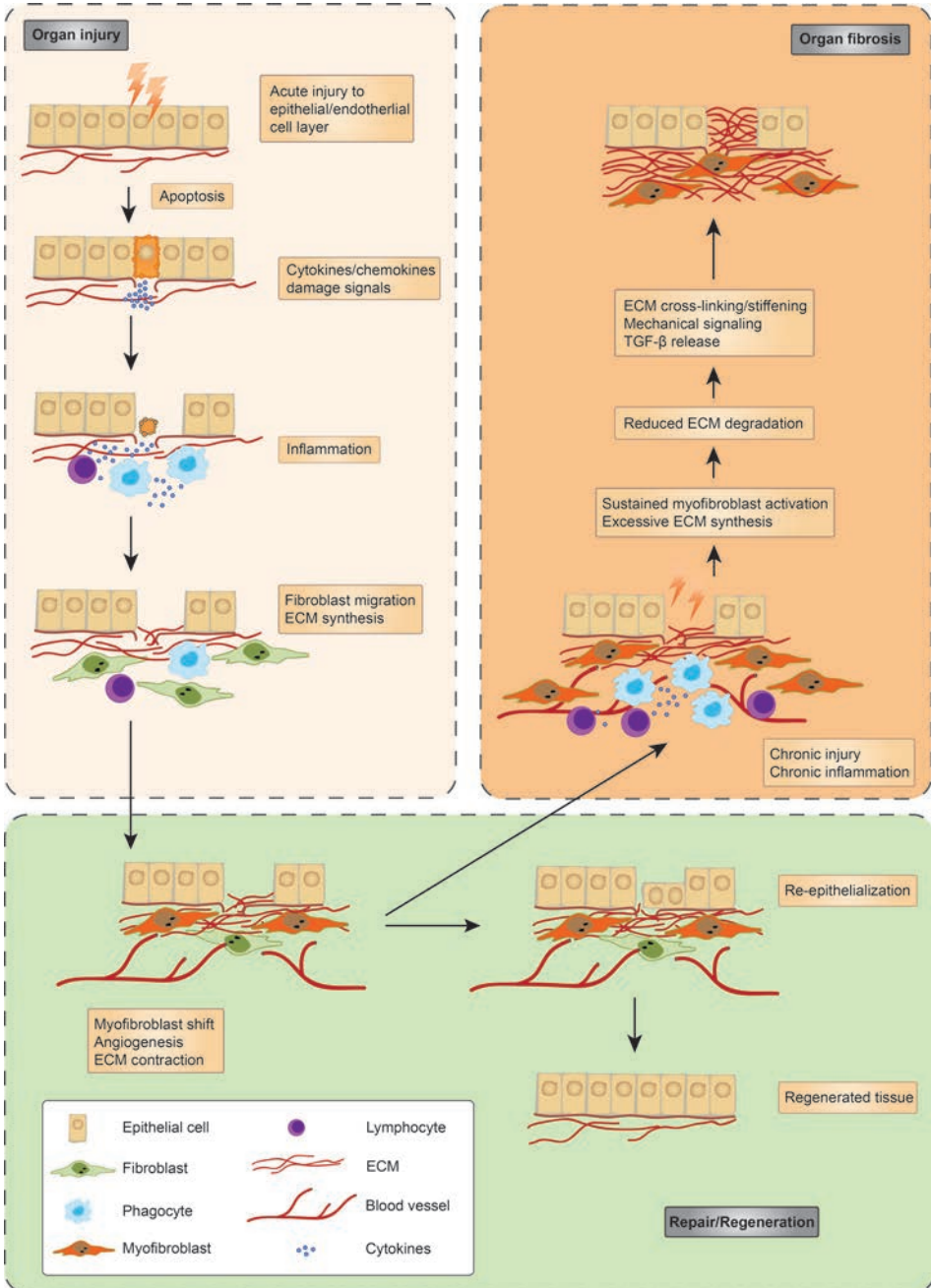
Figure 2. Schematic representation of the wound healing response and the progression towards organ fibrosis.

Through specialized integrin focal adhesions, myofibroblasts connect their actin cytoskeleton to the surrounding ECM and thereby transduce contractile forces into the environment. This results in straining and compaction of ECM molecules, which in turn mechanically feedback to enhance the pro-fibrotic phenotype[17]. Additionally, the mechanical properties of the ECM itself are thought to guide load-bearing collagen assembly via the interactions with fibronectin.

Another form of fibrous tissue can be found in and around several forms of cancer, termed tumor desmoplastic stroma. Desmoplastic tumors are characterized by foci of active myofibroblasts together with increased deposition and an altered organization of ECM proteins[18,19]. Unlike organ fibrosis, desmoplastic stromal ECM is thought to be synthesized by cancer associated fibroblasts (CAFs), as a result of the dysregulated cytokine expression by cancer cells[20]. Recent findings indicate that the desmoplastic stroma is not just an integral part of the tumor but progressively contributes to tumor cell oncogenicity and dissemination[21–24]. ECM in the tumor stroma is thought to influence both the biochemical and biomechanical signaling. Not only is ECM-related mechanosignaling thought to promote the aggressiveness of existent tumor cells, but also promote the transformation of normal epithelial cells into myofibroblast-like cells that support tumor growth, compromise drug delivery, and impair the infiltration of anti-tumor inflammatory cells[25,26].

Despite decades of research, both fibrosis and fibrotic tumors have abysmal options for treatment[27–29]. In the past, the main focus in the development of therapies for these pathologies lay in manipulating biochemical signaling cascades without taking the role of the ECM and the extracellular environment into account. The notion that ECM is of paramount importance in the pathophysiology and thus in the development of effective therapies, has spawned an entire field of research dedicated to ECM-related disorders. Understanding the molecular processes that underlie the pathological accumulation of ECM and consequential signaling through the ECM in both fibrosis and cancer will aid in the development of anti-fibrotic and anti-cancer therapies.

ECM AS SIGNAL TRANSDUCER IN FIBROSIS

Biochemical signaling
Cells react to signals from the environment using different mechanisms, including biochemical and biomechanical cues. The studies on biochemical signaling have their roots in endocrinology studies of the 1970's, when Martin Rodbell first described the actions of the hormone glucagon on guanosine triphosphate (GTP) binding proteins—so called G-proteins—and their associated G-protein coupled receptors[30]. Since then several tens of signal transduction pathways have been uncovered and depending on the cell type, may affect metabolism, cell division, growth, shape and motility. Biochemical signal transduction begins when an extracellular signaling molecule, known as ligand, binds to a membrane bound receptor. One group of ligands for such receptors is the ECM itself. Moreover, ECM often acts as a depot for signaling ligands

including hormones, cytokines, growth factors, signaling ions, and free radicals. The controlled release of these agents allows for a strictly controlled regulation of receptor activation and subsequent signal transduction. Many growth factors are synthesized as an inactive precursor form, transported to the extracellular space and subsequently stored in the ECM. When released by either mechanical or proteolytic means, they can activate cell surface receptors and initiate an intracellular signaling cascade.

The pro-fibrotic TGFβ1 is an example of a growth factor that is kept inactive in the ECM through its association with the latency associated protein (LAP) and latent TGF-binding proteins (LTBPs), together forming the large latency complex (LLC)[31–34]. The LLC is capable of binding to several ECM components such as fibrillins and vitronectin, providing a growth factor reservoir. Latent TGFβ1 is freed from the ECM via several mechanisms: proteolytic cleavage by metalloproteinases (MMPs) and glycosidases, or mechanical liberation through association with integrins. The RGD motif in the LAP is being recognized by the integrins αvβ5, αvβ3, and β1[32]. Upon mechanical strain, the conformation of the complex changes, thereby releasing the TGFβ1 dimer[34,35]. Myriad studies have implicated the actions of TGFβ1 in the processes of wound healing, fibrosis and cancer, but although the basic cascade is similar in all, cell-type specific expression patterns together with inter-pathway cross-talk result in drastically different outcomes[36,37]. For instance, TGFβ1 has been shown to be anti-proliferative in epithelial cells[38], whereas it increases the proliferative capacity of fibroblasts[39,40]. Moreover, studies in cancer suggest that TGFβ1 can act anti-proliferative in the early stages of tumor growth. However, deregulated TGFβ1 signaling has also been shown to boost cancer cell proliferation directly, or via alteration of the microenvironment[41].

Inside the cell, growth factor receptors transduce the signal toward the nucleus. Often, multiple membrane-bound and cytosolic proteins aggregate at the receptor(s) to form signaling complexes[42]. Activated receptor complexes induce post-translational modifications on cytosolic signaling proteins, of which phosphorylation by kinases is the most well-known. Some growth factor signaling cascades activate signaling molecules that directly translocate to the nucleus where they bind to DNA and act as transcription factors and modulate gene transcription. Other signaling cascades have multiple intermediate signaling proteins that act as transduction relay for the signal to travel into the nucleus. In the case of TGFβ1, interaction with its membrane-bound receptors initiates a signaling cascade that activates the signal-propagating complex of Smad transcription factors. Phosphorylated Smad2 and Smad3 complex with Smad4 and translocate to the nucleus, where they associate with DNA-binding proteins or the DNA itself, respectively[37]. Smad3 and Smad4 contain an evolutionary conserved Mad homology (MH1) domain that recognizes an 8-bp palindromic DNA sequence, GTCTAGAC. Signal propagation of Smads depend on the accessibility of chromatin and availability of co-activators, co-repressors, and master transcription factors such as Sox2 and Oct4[43].

Mechanical signaling

Throughout mammalian life, an organism is constantly being subjected to mechanical forces such as pressure force resulting from earth's gravity. Apart from external forces, the body experiences multiple internal mechanical stimuli, including hydrostatic and cellular compression, fluid shear stress, tensional force, and cell traction force, all having profound effects on cells and the ECM. For instance, during human development, the cells of the inner cell mass are being pushed outwards by the excreted liquid in the blastocoel cavity. These mechanical cues cause transcription factors such as Sox2 and Oct4 to be upregulated to maintain pluripotency, which suggest that the embryonic transcriptional program can be influenced by mechanical forces[44]. Moreover, epithelial and endothelial cell layers exhibit pronounced apical-basolateral polarity due to the mechanical signaling of fluid shear stress and constriction on the apical side together with integrin-mediated traction and compression from the basement membrane at the basolateral side. Other mechanical rheostats put a halt on the cell division machinery to make sure organs stop expanding at the right moment in development[45,46].

Cells are able to sense and respond to their mechanical environment through specialized structures called focal adhesions. The term sensing is used metaphorically and refers to extracellular features that can measurably alter a cell's dynamics, function, shape or fate[47]. Focal adhesions are cell-matrix adhesion sites that function as relays between the ECM and the intracellular cytoskeleton. More specifically, focal adhesions are the subcellular structures that regulate the mechanical connection and signaling from and to the ECM, and at the same time function as a biochemical hub for the assembly of multiple signaling proteins at sites of integrin binding and clustering. Lacking kinase activity, integrins mainly act as scaffolding proteins for the recruitment of a wide variety of signaling modules. To discriminate between different ECM components, integrins have the ability to associate as different heterodimers containing an α and β subunit. Mammals express 18 α subunits and 8 different β subunits, which can dimerize and form 24 different integrin receptors[48]. When integrin subunits engage their ligand, talin and kindlin proteins binds to the β-integrin subunit cytoplasmic tail and initiate a conformational change that in turn enhances the affinity for its ligand[49]. These short-lived adhesions, often termed 'nacent adhesions' only last for about 60 seconds, unless they mature into focal complexes. In the latter case, the binding of talin and kindlin triggers the sequestering of multiple scaffolding and signal transduction proteins including filamin, paxillin, vinculin, α-actinin, integrin-linked kinase (ILK), and focal adhesion kinase (FAK)[49]. Together, the aggregated proteins link the integrins with the actin cytoskeleton, and allow bidirectional transduction of mechanical forces.

Actin stress fibers can be typically divided into three groups—dorsal stress fibers, transverse arcs, and ventral stress fibers—that all have a specific function and intracellular localization[50]. Dorsal stress fibers are long, non-contracting bundles of actin that are cross-linked by α-actinin and span the dorsal side of a cell. Transverse arcs are described as long, curved actin bundles with alternating α-actinin and myosin bands, and are not connected to any focal adhesions, but move centripetally along the dorsal stress fibers toward the center of a cell. Finally, ventral stress fibers have

the characteristics of both dorsal stress fibers and transverse arcs. They span the cell as thin linear bundles where they are connected to focal adhesions, but toward the center they contain repetitive stretches of α-actinin and non-muscle myosin II that allows bidirectional contraction. When bipolar myosin motors move along the actin fibers, α-actinin interlocks adjacent actin fibers together to maintain tension[51]. The increased tension between the ECM and the actomyosin cytoskeleton—commonly in the form of ventral stress fibers—initiates clustering of integrins to form the larger focal adhesions[52]. Additionally, it is thought that increased actomyosin-generated forces result in a cascade of protein unfolding events. Such conformational changes could expose otherwise obscured binding sites, consequently modulating the recruitment of additional proteins to the adhesion[47].

Transmission of mechanical cues via integrin receptors sets in motion a cascade of conformational changes that allow a multitude of signaling proteins to aggregate at focal adhesions. Many of these proteins function as signal transducer and possess kinase activity to phosphorylate their substrates, often proteins that also fulfill functions in biochemical signaling cascades. Mechanical signals can alter the concentration of second messengers such as Ca^{2+}, inositol triphosphate, cyclic AMP and nitric oxide, or activate signaling proteins, such as MAP kinases and Rho-family small GTPases[44]. The activation of such signaling cascades lead to changes in the activity of transcriptional modulators, and as such, regulate the transcription of multiple genes. Another example of how mechanical cues modulate transcriptional programs is the activation of the transcriptional co-activators YAP and TAZ, the output of the Hippo signaling cascade. Both YAP and TAZ are actively sequestered in the cytoplasm when the Hippo pathway is active, by means of phosphorylation by the core kinase complex, and association with 14-3-3 proteins at cellular junctions. When the core kinase complex—consisting of LATS1/2, MST1/2, SAV and MOB—is inactivated, YAP and TAZ cannot be phosphorylated. Upon dephosphorylation, YAP and TAZ translocate to the nucleus where they associate with transcription factors, including TEAD's, to modulate transcription of target genes. Besides being subjective to biochemical modulation, YAP and TAZ have been shown to be mechanosensitive[45]. Although the exact mechanism remains elusive, it is thought that actin polymerization is the key factor for YAP and TAZ nuclear translocation[53].

Transduction of mechanical signals often utilizes the same intracellular protein complexes as biochemical signaling cascades. As such, biomechanical and biochemical signaling converge in the activation of transcriptional modulators such as transcription factors, chromatin remodeling enzymes and a multitude of co-activators and co-repressors[43]. Recently, several studies indicated that YAP and TAZ share signaling characteristics with the TGFβ signaling pathway[54–57]. Understanding these interactions and how they modulate the myofibroblast phenotype is crucial for the development of therapies that target the accumulation of ECM in fibrosis.

CHAPTER 1

OUTLINE OF THIS THESIS

Despite decades of intensive research, attempts in developing effective therapeutics against ECM-related disorders, including fibrosis, have largely failed[25,27,29]. The disappointing results from clinical trials targeting the myofibroblast or the fibrotic tissue itself suggest that the nature of the stromal response is far more complex than expected. An explanation for this discrepancy is the lack of understanding of biomechanical signaling and the communication with biochemical signaling cascades. This demands for thorough investigation of the myofibroblast phenotype and the pathophysiology of fibrotic disorders. **The overall aim of this work is to uncover yet unknown biochemical and biomechanical signals that regulate the myofibroblast phenotype in fibrosis and to open up new avenues for development of anti-fibrotic therapies.**

This thesis focuses on several aspects of biochemical and biomechanical activation of myofibroblasts in the context of tissue fibrosis. As discussed above, YAP has recently been discovered to function as mechanical rheostat in mesenchymal stem cells. In **Chapter 2** we sought to delineate the functions of YAP in dermal fibroblasts and in the pathology of a fibroproliferative disorder of the palmar fascia: Dupuytren disease. We investigated whether YAP deficient fibroblasts are still able to phenotypically switch to myofibroblasts upon stimulation with the pro-fibrotic cytokine TGFβ1. Additionally, we investigated the expression and localization profile of YAP in Dupuytren disease biopsies.

The past decade saw a shift in how scientists view biochemical signaling cascades. Instead of regarding them as isolated entities, they were found to interconnect and form complex networks that govern a variety of cellular functions. **Chapter 3** summarizes the current knowledge on how the TGFβ, WNT and YAP/TAZ signaling cascades interact on multiple levels of cellular homeostasis, and how these interactions may drive the initiation and progression of fibrosis.

Chapter 4 forwards on the interaction between TGFβ/Smad signaling and YAP, and shows how YAP regulates the myofibroblast phenotype both on protein and gene level. Furthermore, we show how the actin cytoskeleton regulates the nuclear accumulation of YAP on TGFβ1 exposure, and how this is partly dependent on YAP/Smad interactions. Moreover, we describe how the benzoprophyrin derivative verteporfin inhibits both YAP and Smad2/3 nuclear accumulation, and the expression of signature myofibroblast genes, opening up new avenues for anti-fibrotic therapies.

Spectrins are specialized scaffolding proteins that play a crucial role in maintaining plasma membrane integrity, and have been found to provide mechanical stability to cells. In **Chapter 5** we focus on the role of spectrin proteins in mechanosensing and the myofibroblast phenotypical switch of fibroblasts.

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L-ascorbic acid, commonly referred to as vitamin C, acts a co-factor in eight different types of enzymatic reactions, three of which are crucial in the biosynthesis of collagen. **Chapter 6** describes how vitamin C regulates the biosynthesis of collagens and how this is linked to TGFβ1 exposure and the myofibroblast phenotype.

In **Chapter 7** we discuss how the abovementioned biochemical and biomechanical cues—YAP, spectrins, and vitamin C—may affect the development of ECM related disorders. Finally, we conclude with a discussion of the clinical and therapeutic implications of our findings.

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