Chapter 1
General introduction

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Fibrosis represents an important component of many lethal diseases and can occur in various organs and tissues. In the lungs the most common form of fibrosis is idiopathic pulmonary fibrosis (IPF), where progressive fibrosis leads to loss of respiratory function and eventually death. The incidence of IPF is estimated to be 5 to 10 per 100,000 (Fernandez Perez et al., 2010) and appears to increase in recent years (Nalysnyk et al., 2012). Most IPF patients die within two to five years after diagnosis. Considering the five-year survival of about 20%, IPF has a higher mortality than many cancers. Unfortunately, the current knowledge of the mechanisms involved in the development of lung fibrosis has proven insufficient to develop effective treatment strategies (Adamali and Maher, 2012; Rafii et al., 2013).

Extracellular matrix changes during lung fibrosis

In fibrosis tissue architecture and function are affected by the deposition of large amounts of abnormal extracellular matrix. In healthy lung tissue, the extracellular matrix is rich in collagen, to provide tensile strength, and elastin, to ensure recoil at expiration (Mariani et al., 1997). These and other extracellular matrix proteins are expressed and deposited during development of the lungs. The turnover in healthy adult lung tissue is surprisingly low. However, in disease situations, such as inflammation, wound healing, and fibrosis, a high remodeling may occur, including renewed expression of matrix proteins. Classically, the main problem in fibrosis has been considered to be the overproduction of type I collagen, shifting the proportions of the different matrix proteins. This results in chemical and mechanical changes within the extracellular matrix. The protein content of fibrotic matrix is very different from that of healthy tissue. Furthermore, fibrotic tissue is considerably stiffer than healthy tissue (Booth et al., 2012; Ebihara et al., 2000; Liu et al., 2010).

Fibroblasts: cells of the extracellular matrix

In all living tissues, cells are the active players capable of changing composition, architecture and function of the tissue, including its extracellular matrix. Fibroblasts are the cells specialized in the maintenance of the extracellular matrix. During fibrosis, fibroblasts differentiate into myofibroblasts that are characterized by the expression of increased levels of extracellular matrix proteins and α-smooth muscle actin (αSMA) (Hinz, 2007; Hinz et al., 2012; Tomasek et al., 2002). These characteristics are important in the physiological role of myofibroblasts during wound healing, where these cells fill and close a wound. However, continuing presence of myofibroblasts results in changes in the tissue detrimental to tissue functioning.

Cell matrix interaction

In principle, all nucleus-containing cells in an organism have the same toolbox: the genetic information stored in their DNA. Which tools are activated depends essentially on the signals the cell receives from its environment. For fibroblasts, this environment is largely dictated by the extracellular matrix, which determines chemically and mechanically the surroundings of these cells. Cell surface receptors, such as integrins, allow cells to probe their environment and respond to the composition of the extracellular matrix (Huang and Ogawa, 2012; Wells, 2013). Interestingly, the extracellular matrix is also produced by the cells, or their ancestors, resulting in an intriguing interplay between cells and matrix, where changes in one of the players will affect the other.

Myofibroblast differentiation

The differentiation of fibroblasts into myofibroblasts is under the control of both chemical and mechanical factors in their surroundings. Amongst the chemical factors, the cytokine transforming growth factor (TGF)β_1 is most thoroughly investigated. This cytokine is a remarkable example of the importance of extracellular matrix composition in myofibroblast differentiation and thus fibrosis development. Firstly, TGFβ_1 is sequestered in a latent complex attached to the extracellular matrix and matrix composition will therefore determine the storage capacity for TGFβ_1 within the tissue. Secondly, the mechanical properties of the matrix are central in the release and activation of TGFβ_1 (Wipff et al., 2007).

A second important requirement for myofibroblast differentiation is a certain level of cytoskeletal tension (Tomasek et al., 2002). The buildup of this cytoskeletal tension within the cell requires strong attachment to an extracellular matrix with sufficient stiffness. And again, both the availability of specific attachment sites and the stiffness of the matrix are determined by its composition.

Tackling the problem at the matrix level

The unsuccessful development of treatments for fibrotic diseases such as IPF has largely been focused at interrupting the fibrotic process at the level of chemical, soluble factors. Considering the emerging evidence for a regulatory role of the extracellular matrix in the development of fibrosis, it may be interesting to approach the extracellular matrix molecules as a target for therapeutic intervention. So far, few therapies are aimed at the matrix level, although several drugs acting on varying primary targets have matrix-related side effects (Järveläinen et al., 2009). To be able to design treatment for fibrotic diseases aimed at the extracellular matrix, we need to know more about the reciprocal relationship between matrix and cells in fibrosis. Therefore, in this thesis we set out to investigate several aspects of this intricate relationship between matrix and cells in an effort to gain

more insight into mechanisms in the development of lung fibrosis that could help in future development of treatment.

Approach and outline

In patients, pulmonary fibrosis is usually diagnosed quite late, due to the large overcapacity of the lung tissue. Therefore, research on the early stages of the development of fibrosis relies heavily on animal models. In the most commonly used model, pulmonary fibrosis is induced by instillation of bleomycin into the lungs of rodents (Moore et al., 2013). This results in the formation of reactive oxygen species within the lung, causing damage to tissue and cells. As a response, inflammation and fibrosis occur within the lung tissue. To specifically address the development of lung fibrosis *in vivo*, we follow mice at 1-week intervals during a period of 5 weeks after induction of fibrosis. In the model, we determine new collagen formation, the core process of fibrosis, using a state-of-the-art technique, measuring deuterated water incorporation into hydroxyproline, an amino acid mainly present in collagen. Next, we correlate this new collagen formation to gene expression data from microarray analysis. This way we attempt to identify fibrosis-relevant genes and pathways, described in **chapter 2**.

In **chapter 3** we zoom into three of the fibrosis-relevant genes, encoding for the extracellular matrix proteins elastin, type V collagen and tenascin C, to further investigate the role of these three proteins in the development of lung fibrosis. Here we first study in more detail their expression during the development of bleomycin-induced lung fibrosis. Next, we study whether the fibroblasts are the cells responsible for the changed expression of these extracellular matrix proteins. For type V collagen and tenascin C, regulatory roles in fibrosis development have been suggested in the literature (Braun et al., 2010; Tamaoki et al., 2005). Since this is not known for elastin, we continue with investigating the effect of the observed increased elastin expression on lung fibroblasts.

During the development of lung fibrosis, the lung tissue becomes considerably stiffer (Booth et al., 2012; Ebihara et al., 2000; Liu et al., 2010). This might influence the amount of stretch experienced by the resident lung fibroblasts during the breathing cycle. In **chapter 4**, our aim was to study the effect of cyclic mechanical stretch, mimicking the breathing movement, on gene expression of fibrosis-relevant genes in human lung fibroblasts *in vitro*. In this way we attempt to investigate whether fibrosis-related stiffening of lung tissue influences myofibroblast differentiation and thus disease progression.

One interesting candidate protein that could connect cells, matrix and mechanics at a molecular level is caveolin. Caveolin-1 is a protein located in the plasma membrane and the structural component of caveolae – plasma membrane invaginations associated with

several extracellular matrix-relevant cellular processes. Caveolin-1 interacts with a variety of cell surface receptors, amongst which the integrins, important for attachment and communication of the cell to the extracellular matrix. Furthermore, caveolin-1 and caveolae have been described to play a role in mechanotransduction, allowing cells to respond to changes in the stiffness of the extracellular matrix. Thus, caveolin-1 and caveolae play an important role in the response of cells to fibrosis-related changes in the extracellular matrix. Furthermore, an intricate relationship between caveolin-1 and $TGF\beta_1$-signaling completes the circle of extracellular matrix changes and myofibroblast differentiation during fibrosis. Indeed, caveolin-1 has been associated with pulmonary fibrosis (Tourkina et al., 2008; Wang et al., 2006). Therefore, in **chapter 5** we review the literature in order to collect evidence on possible mechanisms for the role of caveolin-1 in fibrotic diseases.

In **chapter 6** the information gathered in this thesis is integrated into a general discussion on the reciprocal relationship between cells and matrix during the development of lung fibrosis.

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