

General discussion



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Fibrosis is an important component of various lethal diseases. Currently, no effective treatment is available, possibly due to the classical approach aiming at interrupting cellular signaling events at the level of soluble factors. However, fibrosis is characterized by changes in the extracellular matrix. Since the extracellular matrix forms the environment of cells and can thus determine cellular behavior, we investigated the interactions between the cells and matrix during the development of lung fibrosis in an effort to find leads that could help in the search for alternative treatment approaches.

We started with an in-depth analysis of the development of lung fibrosis in the murine model of bleomycin-induced lung fibrosis. In this model, we analyzed the deposition of new collagen and correlated this to gene expression data from microarray analysis, thereby creating a gene expression signature of fibrosis within the lung. Additionally, functional grouping of genes provided us with information about cellular pathways regulated during active fibrosis. After this, we zoomed in on three extracellular matrix proteins highly correlated with new collagen deposition: elastin, type V collagen and tenascin C. We found increased expression of all three matrix proteins in histological sections from the same animals. Furthermore, *in vitro* experiments indicated that transforming growth factor (TGF) β_1 -induced fibroblasts-to-myofibroblasts differentiation increased gene expression levels of these three extracellular matrix proteins. In turn, elastin increased fibroblast-to-myofibroblast differentiation. Next, we investigated the effect of a possible reduction of cyclic mechanical stretch reaching the fibroblasts in fibrotic tissue, due to the increased stiffness of the tissue. We found that cyclic mechanical stretch mimicking breathing in healthy lung tissue, resulted in a reduction of fibroblast-to-myofibroblast differentiation. Levels of the extra domain A (ED-A) splice variant of fibronectin did not change, but the reduction of paracrine expression of TGF β_1 suggests a possible regulatory mechanism. Finally, we reviewed the literature on a possible role for caveolin in the development of fibrosis, since caveolin can influence TGF β signaling, cell matrix interaction, and the sensitivity of cells to chemical and mechanical cues, thereby taking a central spot in many fibrosis-relevant cellular processes. Below, we will discuss these findings in the wider context of cell matrix interaction during the development of fibrosis.

Fibrosis changes the chemical properties of the matrix

During the development of lung fibrosis the extracellular matrix changes. To obtain an overview of the changes in gene expression, we performed a microarray analysis on lungs from mice with bleomycin-induced lung fibrosis (**chapter 2**). We focused on the genes of which the expression was highly correlated with fibrosis activity, as measured by the deposition of newly formed collagen. In mice with active fibrosis, it appeared that many genes related to the extracellular matrix were upregulated. Some of these proteins are

related to collagen deposition, such as thrombospondin 2, playing a role in fibril formation, and lysyl oxidase, important for the crosslinking of collagen. The collagen degrading enzyme matrix metalloproteinase 14 was upregulated as well, indicating an increased remodeling activity. Furthermore, three extracellular matrix proteins other than type I collagen were strongly upregulated in mouse lungs with high fibrotic activity: elastin, tenascin C and type V collagen. In histological sections of the same mice we confirmed the upregulation of these three extracellular matrix proteins at the protein level (**chapter 3**). While elastin staining continued to increase at least 5 weeks after fibrosis induction, tenascin C and type V collagen staining was initially increased and started to decrease after 3 weeks. This is likely related to the transient nature of fibrosis induced by a single dose of bleomycin (Chua et al., 2005; Moeller et al., 2008). Upregulation of these genes in human lung fibroblasts cultured *in vitro* under fibrotic conditions, i.e. in the presence of TGF β ₁, provides evidence that fibroblasts contribute to this increased expression *in vivo*.

Thus, during the development of lung fibrosis, expression of different extracellular matrix proteins is increased, such as elastin, type V collagen and tenascin C (**chapter 3**). Interestingly, expression and deposition of these and other extracellular matrix proteins are very low in healthy adult tissue, while high expression levels are critical during development (Chiquet-Ehrismann and Chiquet, 2003; Jones and Jones, 2000; Mariani et al., 1997; Powell and Whitney, 1980; Roth-Kleiner et al., 2004; Roulet et al., 2007). This suggests that during fibrosis a developmental program of gene expression is reactivated (Shi et al., 2009). Unfortunately, this reactivation of developmental gene expression does not result in redevelopment of a properly structured extracellular matrix but leads to improperly functioning lung tissue. It would be interesting to determine the differences between expression patterns during development and during fibrosis, although this is probably difficult to establish, as minor differences in timing could already have drastic consequences in the final resulting tissue.

Fibrosis changes the mechanical properties of the matrix

Next to the chemical properties, also the mechanical properties of the lung tissue change during the development of fibrosis. The most recognized mechanical change is the increased stiffness of fibrotic lung tissue (Booth et al., 2012; Ebihara et al., 2000; Liu et al., 2010). This is in part due to the deposition of large amounts of unstructured extracellular matrix proteins. However, also changes in the crosslinking of the matrix molecules will influence tissue stiffness (Georges et al., 2007; Norton et al., 1997). This phenomenon is observed in lung as well as other types of fibrosis (Brinckmann et al., 2001; Georges et al., 2007; Hayasaka et al., 1996; Last et al., 1990). In this context, it is interesting to note that in mice with active fibrosis we observed an upregulation of lysyl oxidase (**chapter 2**), an enzyme necessary for the formation of crosslinks of collagen and elastin (Eyre et al., 1984).

This suggests that in bleomycin-induced lung fibrosis increased crosslink formation plays a role in the increased stiffness of the lung tissue as observed by (Liu et al., 2009).

The chemical and mechanical properties of the matrix influence cell behavior

Classically, changes in the extracellular matrix have been considered purely as the final result of fibrosis. In recent years, a much more active role for the extracellular matrix in fibrosis is starting to emerge (Klingberg et al., 2013; Wight and Potter-Perigo, 2011). Both the chemical and mechanical properties of the extracellular matrix can influence cellular processes relevant for the development of fibrosis, such as myofibroblast differentiation. A convincing example of the influence of a fibrotic extracellular matrix on cell behavior was given by Booth and colleagues (Booth et al., 2012). After seeding primary human lung fibroblasts into decellularized lung matrices of IPF patients and healthy controls, they found that extracellular matrix from IPF lungs resulted in increased levels of the myofibroblast marker α -smooth muscle actin. Acquiring knowledge on which properties of the extracellular matrix are responsible for this pro-fibrotic matrix effect will be important in our search for matrix-targeting treatment options.

The chemical properties of the matrix can play a regulatory role in fibrosis, because many of the proteins shown to be present at increased levels in the fibrotic extracellular matrix have the capacity to influence fibrosis-relevant cellular processes. In **chapter 3** we found a direct stimulatory effect of extracellular elastin on myofibroblast differentiation. Type V collagen might also have pro-fibrotic effects, because availability of this type of collagen results in an inflammatory response (Braun et al., 2010) and inflammatory cells excrete growth factors important in myofibroblast differentiation, such as TGF β ₁ (Todd et al., 2012). Tenascin C increases cell migration (Trebaut et al., 2007), important for myofibroblast recruitment. Hence, changes in the composition of the extracellular matrix during the development of fibrosis enhance myofibroblast availability and thus disease progression.

The mechanical properties of the fibrotic extracellular matrix also have the capacity to affect cellular behavior. Increased stiffness of the extracellular matrix allows cells to build up more cytoskeletal tension, an important factor in myofibroblast differentiation (Goffin et al., 2006; Huang et al., 2012; Li et al., 2007). Furthermore, stored latent TGF β ₁ will be more easily released and activated from a stiffer matrix (Wipff et al., 2007). Besides the direct effect of tissue stiffness on cells, the mechanical properties of the matrix also determine the exposure of resident cells to external mechanical loading, such as the stretch of tissue during breathing. This further influences cell behavior. Indeed, in **chapter 4** we found that cyclic mechanical stretch, mimicking the breathing movement, could help keeping the fibroblasts healthy, since it reduced expression of myofibroblast-related genes.

Fitting the pieces together: Towards a model for the development of lung fibrosis

From the above we can conclude that during the development of lung fibrosis, the cells and the matrix form an interactive system. This reciprocal interaction can result in a positive feedback loop, amplifying disease progression once a certain threshold change in any of the components has been reached (Figure 1). This provides us with a model that could help us understand the progression of lung fibrosis. In order to develop new effective therapies, we should search for the component or components within the system that is most sensitive to change by therapeutic means. In a positive feedback loop the process can be interrupted in any of the components, because reversing the changes in one component will automatically halt changes in the other component.

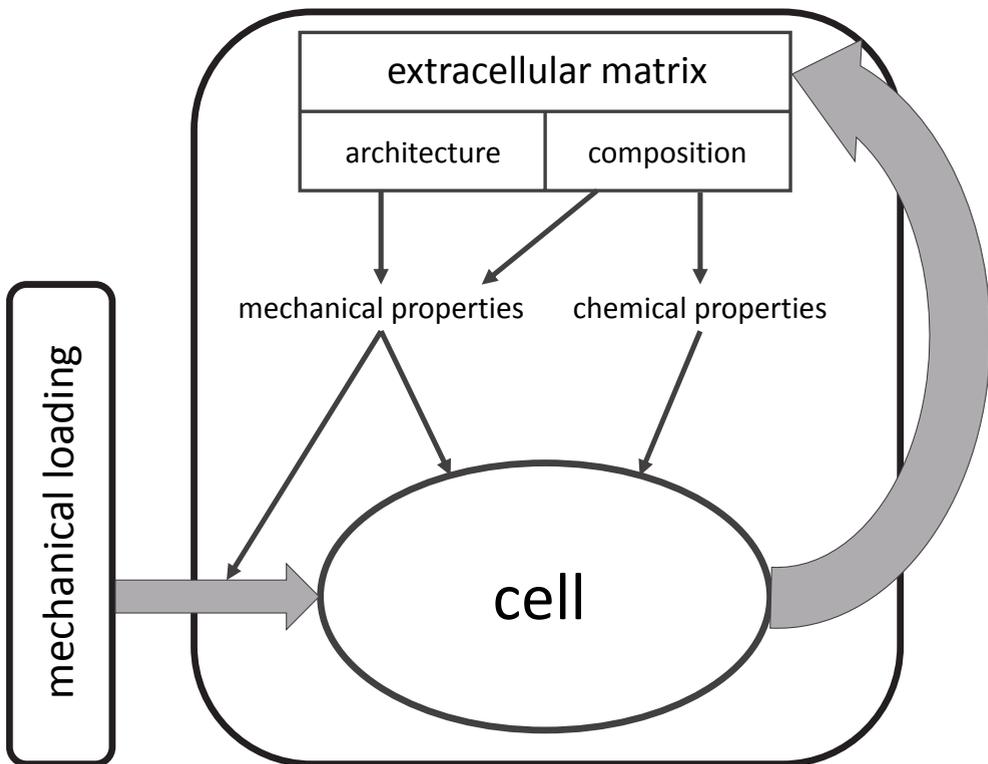


Figure 1: Overview of cell matrix interactions during the development of lung fibrosis. The cell changes the chemical properties of the matrix, mostly determined by its composition, and the mechanical properties, determined by both the composition and the architecture of the matrix. Reversely, both mechanical and chemical properties of the extracellular matrix influence cell behavior. Mechanical properties of the matrix determine also the exposure to external mechanical loading, such as occurring during breathing. This interactive system contains positive feedback loops that could explain the progressive nature of lung fibrosis and provides us with a theoretical framework for future therapeutic developments against lung fibrosis.

Table 1: Extracellular matrix changes during lung fibrosis and their proposed pro-fibrotic activity

Chemical changes	Regulation in human lung fibrosis	Proposed pro-fibrotic activity
Matrix proteins		
Type I collagen	Upregulated (McDonald et al., 1986)	
Type V collagen	Upregulated (Parra et al., 2006)	Evokes inflammatory response (Braun et al., 2010)
Elastin	Upregulated (Cha et al., 2010)	Increases myofibroblast differentiation (this thesis)
Fibronectin ED-A	Upregulated (Kuhn et al., 1989)	Necessary for myofibroblast differentiation (Serini et al., 1998)
Matrixcellular proteins		
SPARC	Upregulated (Kuhn and Mason, 1995)	Increases epithelial cell damage (Shibata and Ishiyama, 2013) Modulates procollagen processing (Harris et al., 2011)
Osteopontin	Upregulated (Pardo et al., 2005)	Increases fibroblast proliferation & migration (Pardo et al., 2005)
Thrombospondin	Upregulated (Kuhn and Mason, 1995)	Decreases MMP2 and MMP9 (Maclauchlan et al., 2009; Yang et al., 2000) Increases fibroblast adhesion (Kyriakides et al., 1998; Yang et al., 2000)
Tenascin C	Upregulated (Fitch et al., 2011; Kuhn and Mason, 1995)	Increases fibroblast contraction (Kyriakides et al., 1998; Maclauchlan et al., 2009)
CCN2/CTGF	Upregulated (Kono et al., 2011)	Increases myofibroblast recruitment (Imanaka-Yoshida et al., 2001) Increases epithelial-mesenchymal transition (Sonnylal et al., 2013) Increases fibroblast proliferation & extracellular matrix synthesis (Frazier et al., 1996)
CCN4/WISP-1 periostin	Upregulated (Konigshoff et al., 2009) Upregulated (Naik et al., 2012; Okamoto et al., 2011)	Increases myofibroblast differentiation (Konigshoff et al., 2009) Promotes secretion of extracellular matrix (Naik et al., 2012)
Mechanical changes		
direct		
stiffness	Increased (Booth et al., 2012; Ebihara et al., 2000; Liu et al., 2010)	Increases cytoskeletal tension Increases TGFβ1 release and activation (Wipff et al., 2007)
indirect		
cyclic mechanical stretch	Reduced	Increases myofibroblast differentiation (this thesis)

In literature, evidence is accumulating for other possible feedback loops that may be active during the development of fibrotic diseases. Besides the classically described increase in the expression of type I collagen (McDonald et al., 1986), also fibronectin containing extra type III domain A (ED-A) is found at higher levels in patients with lung fibrosis (Kuhn et al., 1989). Furthermore, fibrotic lungs display a changed expression of several matricellular proteins - non-structural proteins present in the extracellular matrix with various regulatory functions. For both, the matricellular proteins and the structural extracellular matrix proteins that are present at increased levels in fibrotic tissue, fibrosis-relevant regulatory effects have been described. An overview is given in Table 1, which is far from complete, illustrating the large influence of the extracellular matrix on fibrosis-relevant cell behavior.

Possibilities for clinical intervention

Based on the matrix-related changes in fibrotic lung tissue described in this thesis as well as in literature and their regulatory role in lung fibrosis, we can suggest new leads to possible therapeutic interventions. In **chapter 3** we learned that adhesion to elastin makes lung fibroblast more sensitive to TGF β ₁-induced myofibroblast differentiation. This suggests that preventing this adhesion would reduce myofibroblast differentiation in the tissue. Adhesion to elastin can occur via α _v β ₃-integrin (Bax et al., 2009) or heparin and chondroitin sulfate-containing glycosaminoglycans (Broekelmann et al., 2005), possibly dependent on the tissue. Furthermore, there is an elastin receptor complex, also important in elastin assembly (Duca et al., 2007). To be able to target the cell attachment to elastin inducing myofibroblast differentiation, further research needs to specify which adhesion molecule is involved.

Based on our observation that cyclic mechanical stretch mimicking the breathing movement reduced myofibroblast differentiation (**chapter 4**), aerobic training, necessitating heavy breathing, might be an effective preventative measure against lung fibrosis.

Besides these suggestions based on our findings, several interventions targeting the extracellular space are currently under investigation. For example, interfering with cell attachment and adhesion to the extracellular matrix via antibodies against specific integrins is considered as anti-fibrotic treatment (Horan et al., 2008; Puthawala et al., 2008; Rafii et al., 2013; Wang et al., 2000). A major difficulty with this approach is the large chance on side effects, due to the ubiquitous expression and function of integrins in the human body. Furthermore, considering that cytoskeletal tension within the fibroblast is essential for myofibroblast differentiation (Tomasek et al., 2002) and the increased stiffness of fibrotic tissue (Booth et al., 2012; Ebihara et al., 2000; Liu et al., 2010) will increase cytoskeletal tension, it could be effective to therapeutically reduce cytoskeletal tension. This could be achieved by inhibiting Rho-kinase (ROCK). Indeed, the ROCK inhibitors Y-27632 (Shimizu et al., 2001) and fasudil (Jiang et al., 2012) decreased lung fibrosis in bleomycin-instilled

mice. However, in these studies the ROCK inhibitors were administered before fibrosis was established, an approach poorly comparable to patient treatment.

In order to recognize patients in an earlier stage of fibrotic lung disease, it may be useful to develop alternative diagnostic methods besides the currently standard histological qualification. One such approach could be determining levels of certain markers in the circulation. Such an approach has been suggested for systemic sclerosis (Yanaba et al., 2012). These authors found that serum levels of growth differentiation factor-15 (GDF-15) increased with disease severity in systemic sclerosis patients, and especially patients with pulmonary fibrosis had elevated levels. Interestingly, we found that in the lung gene expression of GDF-15 was correlated with fibrosis activity during bleomycin-induced lung fibrosis (**chapter 2**). This indicates that the source of serum GDF-15 is located within the affected tissue, thereby emphasizing the possibility for a regulatory role of GDF-15 and validating this marker for diagnostic purposes. Furthermore, it indicates that our approach in **chapter 2** can provide us with further candidates for circulatory lung fibrosis markers.

There is now ample evidence that there are many different changes in the extracellular matrix that influence cellular processes important in fibrosis. An overview is given in table 1. As a consequence, addressing only one or few of these changes might not be sufficient to stop the circular process of fibrosis. Therefore, as a possible solution, we would suggest focussing further research on caveolin. Caveolin is an integral membrane protein necessary for the formation of caveolae – invaginations in the plasma membrane rich in cholesterol and signaling molecules, such as integrins and the TGF β -receptor. In this position, caveolin can influence TGF β signaling, cell matrix interaction, and the sensitivity of cells to chemical and mechanical cues, thereby taking a central spot in many fibrosis-relevant cellular processes (**chapter 5**).

The start of lung fibrosis: involvement of caveolin?

It is interesting to speculate about what came first, the changes in the matrix or the changes in the cells? The cell will have to receive signals from its environment before it will change, while the matrix usually changes due to changed cellular activity. In our model of bleomycin-induced lung fibrosis, the first stimulus is cell damage since the drug induces the production of reactive oxygen species. It is possible that in patients the fibrotic process is started by a similar primary cause of cell damage, such as mechanical injury, infections, autoimmune reactions, radiation and toxicity caused by exposure to chemicals, e.g. as occurs while smoking (Wynn, 2008). According to the current understanding, IPF results from multiple cycles of epithelial cell damage (Selman and Pardo, 2006). It is noteworthy that in lung fibrosis decreased levels of caveolin-1 have been specifically localized in lung epithelial cells of IPF patients (Wang et al., 2006) and of rats and mini-pigs *prior* to radiation-induced lung fibrosis (Kasper et al., 1998). Decreased levels of caveolin-1 result in decreased numbers of

caveolae. Caveolae are important for their capacity to protect cells from damage when exposed to mechanical stretch, due to their membrane buffering capacity (Sinha et al., 2011). All lung cells are exposed to cyclic stretch during the breathing cycle (Tschumperlin et al., 2010), and thus depend on this membrane buffering by caveolae. Therefore, the reduced levels of caveolin-1 and caveolae in epithelial cells during fibrosis will probably result in increased levels of epithelial cell damage. This might be an important mechanism for the involvement of caveolin-1 in fibrosis.

From here and further

In patients, IPF is diagnosed in a late stage of disease progression, due to the large overcapacity of the lungs. Because of this late diagnosis, the aetiology of IPF is largely unknown and little information is available about the early phases in the development of this type of fibrosis. Therefore, for more insight into these early phases of fibrosis development, research heavily relies on animal models (Chua et al., 2005). In this thesis, we used a mouse model to study the development of lung fibrosis. In this model, fibrosis is induced by a single dose of bleomycin instillation into the lungs. This version of the bleomycin model is known to mimic the progressive nature of human lung fibrosis to a limited extent because in the mouse model fibrosis is transient (Chua et al., 2005; Moeller et al., 2008) and repetitive bleomycin administration has been suggested as a more persistent alternative (Degryse et al., 2010).

It is interesting to consider, however, that it is very well possible that there are also people with a transient, light form of lung fibrosis. These patients might never reach the clinic due to lack of symptoms at this early stage and recover without ever knowing that they had fibrosis. If this is true, only the severe cases not capable to recover present with symptoms in the clinic. We can speculate about two possible differences between the patients with light, resolving fibrosis and severe, progressive fibrosis. Firstly, the progressive fibrosis could be caused by a more severe and/or repeated primary cause, comparable to the model with repetitive bleomycin administration. This might result in changes above a certain threshold of disease development, after which no return is possible. Secondly, the patients suffering from progressive fibrosis might be less capable of resolving fibrosis, due to secondary causes and/or genetic predisposition. Taking these possibilities into consideration, future research on fibrosis should aim at gathering information about the following: (a) What is the key factor determining the point of no return? (b) How could recovery take place: what is able to interrupt the positive feedback loop characterizing fibrosis development? And considering the importance of changes in the extracellular matrix in disease progression, (c) does the extracellular matrix change differently in transient vs. progressive fibrosis?

Since the obvious difficulties in studying this in patients, an important source of information on this could be the comparison between the transient bleomycin model with

the more persistent version of the model, induced by repetitive bleomycin administration. Alternatively, it is interesting to compare the transient bleomycin model with non-recovering patients. In this line, we observed a transient staining of type V collagen and tenascin C in the transient mouse model (**chapter 3**), which is in contrast to the similar staining in end-stage IPF patients (Fitch et al., 2011; Kuhn and Mason, 1995; Parra et al., 2006). Considering the regulatory roles of type V collagen and tenascin C in fibrosis development (Braun et al., 2010; Trebault et al., 2007), it is tempting to suggest that the transient presence of these proteins in the matrix could be the cause of the transient nature of the mouse model. Evidently, further research is needed to investigate these and other differences between transient and progressive fibrosis and determine clear cause-and-result relationships. Altogether, this new perspective on fibrosis may provide us with promising new leads for future research and treatment options for lung fibrosis.

General conclusions

During the development of lung fibrosis fibroblasts change the composition of the extracellular matrix, by an increased expression of extracellular matrix proteins such as elastin, type V collagen and tenascin C. This affects both the chemical and mechanical properties of the matrix. In turn, these properties affect fibrosis-relevant cellular processes, such as myofibroblast differentiation. This reciprocal relationship between the cells and the matrix results in a positive feedback loop, amplifying cell and matrix changes, thus explaining the progressive nature of lung fibrosis in patients. It may be effective to target lung fibrosis at the level of this reciprocal cell matrix interaction. Future studies could focus on caveolin, since this membrane protein is capable of influencing the sensitivity of cells to both the chemical and the mechanical changes in the matrix. Thereby it seems to take a central spot in many fibrosis-relevant cellular processes and could be a promising target for therapeutic interventions.

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