Chapter 1

General introduction & scope of the thesis
COPD

Chronic obstructive pulmonary disease (COPD) is the general term for a number of distinct but often partially overlapping, phenotypes; emphysema, small airway disease and chronic bronchitis. With approximately three million deaths it was the third leading cause of death worldwide in 2016 (1, 2). In the western world, inhalation of irritants, mainly tobacco smoking, is the major risk factor for the development of COPD (3).

COPD patients have a chronic inflammatory response to irritants and a disturbed repair system, which leads to progressive airflow limitation. This airflow limitation is due to a combination of destruction of alveolar septa with lack of repair in emphysema and excessive extracellular matrix (ECM) deposition in the airway walls in chronic bronchitis and small airway disease (4-6). Although the extent of these processes varies from patient to patient resulting in different phenotypes, these eventually lead to a persistent decline in lung function and breathing difficulties even during daily life activities (3).

To determine the lung function of patients, spirometry tests are performed which measure the airflow. The Forced Expiratory Volume in one second (FEV₁) and the Forced Vital Capacity (FVC) are two important parameters of the spirometry tests. A FEV₁/FVC ratio below 70% indicates airway obstruction. The severity of the airflow limitation in COPD patients is classified into four categories based on the FEV₁ according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (Table 1) (7).

### Table 1. Classification of airflow obstruction severity in COPD patients with FEV₁/FVC<70%

<table>
<thead>
<tr>
<th>GOLD Stage</th>
<th>Severity</th>
<th>FEV₁ (% predicted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mild</td>
<td>≥ 80</td>
</tr>
<tr>
<td>II</td>
<td>Moderate</td>
<td>50 – 79</td>
</tr>
<tr>
<td>III</td>
<td>Severe</td>
<td>30 – 49</td>
</tr>
<tr>
<td>IV</td>
<td>Very severe</td>
<td>&lt; 30</td>
</tr>
</tbody>
</table>

*Airway obstruction classification according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (7).

In addition to GOLD stages, the ABCD assessment tool was introduced which takes the symptoms and the exacerbation history of the patient into account (7). The symptoms including dyspnea are measured using several questionnaires (i.e. the Modified British Medical Research Council Questionnaire and the COPD Assessment Test). The lung function combined with the assessment of symptoms and the risk of exacerbations, provides a more representative picture of the disease severity and is considered to be a better guide for treatment decisions.

COPD patients are always advised to change their lifestyle. Smoking cessation, with or without the help of therapeutic intervention, is one of the most important changes that
General introduction & scope of the thesis

COPD and the link with ageing

The incidence of COPD increases with age; the majority of the patients diagnosed with COPD are 60 years or older (10, 11). As COPD is mainly diagnosed in the late middle-aged and elderly individuals, this lung disease has been suggested to be associated with ageing (10, 12). Ageing can be defined as a process in which the homeostasis in the body progressively declines after the reproductive phase (12). Ageing effects can be observed in a normal lung from an age of 25 years and include the gradual loss of its elasticity due to alterations in the ECM (13).

At the molecular and cellular level, several features in the lungs of COPD patients are quite comparable to the features observed in normally ageing lungs of healthy subjects, albeit at an obviously earlier age. These features include increased levels of reactive oxygen species (ROS) and mitochondrial dysfunction, which subsequently lead DNA damage, changes in the DNA methylation pattern, shortening of telomeres, cellular senescence and protein modifications resulting in loss of protein function (10, 14). Both ROS and mitochondrial dysfunction have been described as hallmarks of ageing (15). Other hallmarks of ageing that also have been found to play a role in COPD are deregulated nutrient sensing, reduced repair capacity of basal progenitor cells and altered cellular and intercellular communication (10, 16). Involvement of these different hallmarks underlines the complexity of COPD and the normal ageing process. Unravelling the mechanisms involved in ageing may contribute to a better understanding of COPD and may provide novel therapeutic targets for treating this lung disease.

Lung tissue repair and remodelling

The extracellular matrix (ECM) in the lungs, mainly composed of collagens, elastic fibers, proteoglycans, fibronectin and tenascin, is important for a proper lung architecture (17). Collagen and fibronectin contribute to the tensile strength of the lungs whereas elastin, which is the main component of elastic fibers, is responsible for the elastic recoil (18). In addition to providing structural support and stability to the lung, the ECM also influences the phenotype and behaviour of cells (19). Induction of integrin expression on the cell surface can upon binding to ECM components initiate downstream signalling pathways. In addition, the ECM may also influence growth factor receptor expression patterns in lung can slow down the decline or stabilize the lung function in COPD patients (8, 9). Currently, the pharmacotherapies for COPD only relieve the symptoms and reduce exacerbations. The different phenotypes require personalized medicine to obtain a more effective individual treatment outcome. The mechanisms underlying this incurable lung disease are until now largely unclear. Better insight in the underlying mechanisms is required to find novel therapeutic targets for the development of new treatments.
cells (20). These characteristics emphasize that a proper composition of the ECM is crucial to maintain the normal lung function.

Various cell types in the lung, like airway smooth muscle cells, epithelial cells and (myo)fibroblasts are able to produce ECM proteins. The lung fibroblasts that differentiate into myofibroblasts mainly upon stimulation with transforming growth factor beta (TGF-β) are the main producers of ECM proteins (19). The majority of the lung fibroblasts are located in the airway wall (i.e. airway fibroblasts) and in the interstitium of the lung parenchyma (i.e. parenchymal fibroblasts). During normal tissue homeostasis, old and damaged proteins in the ECM are degraded by matrix metalloproteases (MMPs) and a disintegrin and metalloproteases (ADAMs) (21). These enzymes can be inhibited by tissue inhibitors of matrix metalloproteases 1-4 (TIMP1-4). The degraded, old proteins are replaced by newly produced proteins. As lung fibroblasts can synthesize and secrete ECM proteins as well as MMPs and TIMPs, a proper function of these cells is crucial for controlling ECM homeostasis (19, 22). An imbalance in production and degradation of the matrix of the lung can lead to aberrant tissue repair and remodelling (Figure 1). This is one of the features of the lungs of COPD patients (21). In patients with emphysema, characterized by a destruction of the alveolar walls and lack of repair, the expression levels of MMP-1 and MMP-9 are increased (23). These increased MMP levels may contribute to the shift towards a net ECM degradation. In airway fibrosis, there is an excess of ECM deposition (24).

In addition to the secretory property, lung (myo)fibroblasts also have a contractile function. These contraction forces are important in tissue repair as they mediate re-epithelialization by wound closure (25).

**TGF-β signalling pathway**

TGF-β is produced by alveolar macrophages, epithelial cells and (myo)fibroblasts in the lungs. It is a multifunctional cytokine known to be involved in several processes including cell proliferation, differentiation and tissue repair and remodelling (26, 27). Of the three isoforms known to be expressed in the lungs, i.e. TGF-β1 (Chr 19q13.2), TGF-β2 (Chr 1q41) and TGF-β3 (Chr 14q24.3), TGF-β1 is the best studied isoform. In COPD patients, TGF-β1 has been reported to be elevated in plasma and in small airway epithelial cells (28-30). Parenchymal fibroblasts of COPD patients have been shown to secrete significantly more TGF-β1 compared to those of non-COPD controls (4).
Chapter 1

14

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Figure 1. Aberrant tissue repair and remodelling in the lungs. After TGF-β stimulation, the lung fibroblasts can differentiate into myofibroblasts. Both fibroblasts and myofibroblasts can produce ECM proteins. During normal tissue repair and remodelling, there is a balance in the production and degradation of the ECM. In emphysema there is a destruction of the alveolar walls and lack of repair, while in airway fibrosis, there is an excess of ECM deposition.

TGF-β dimers are secreted in an inactive form that can be activated upon proteolytic cleavage by, for example, integrins and proteases (30). Upon binding to its receptor expressed on fibroblasts, the active form of TGF-β can initiate SMAD-dependent as well as SMAD-independent signalling. Of these two signalling pathways, the SMAD-dependent signalling pathway is considered as the main downstream pathway of TGF-β. This pathway is active upon binding of TGF-β to the type I and type II TGF-β receptor dimers. Subsequently, the type II receptor activates the type I receptor through phosphorylation, which results in phosphorylation of the TGF-β specific SMAD2 and SMAD3 proteins. The phosphorylated SMAD proteins will form a complex with SMAD4 and translocate into the nucleus. This complex binds to SMAD-binding elements in the promoters of TGF-β-specific target genes, which include protein coding and microRNA (miRNA) genes (31).
To control this signalling pathway, the inhibitory SMAD7 forms a complex with Smurfl and Smurf2 ligases which can guide the whole complex to the activated TGF-β type I receptor. The Smurf enzymes degrade the receptor resulting in inhibition of the SMAD-dependent TGF-β signalling pathway.

Significantly decreased protein levels of TGF-β1, TGF-β receptor type I and SMAD7 have been reported in airway epithelial cells and stromal cells in peribronchial area of small airways of stage II COPD patients compared to non-COPD controls (32). In addition, stimulation of lung fibroblasts with tumour necrosis factor and/or cigarette smoke extract significantly reduced SMAD3 and SMAD7 levels. This effect was more pronounced in lung fibroblasts derived from COPD patients compared to those derived from non-COPD controls (33). These findings indicate an altered TGF-β signalling pathway in fibroblasts of COPD patients.

MiRNAs and indications of their role in COPD pathogenesis and ageing

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs with an average length of 22 nucleotides. The transcription of miRNA genes to primary miRNAs (pri-miRNAs) occurs in the nucleus and is executed by RNA polymerase II (Figure 2) (31, 34, 35). The pri-miRNA contains one or more hairpin structures with the mature miRNA sequences being present in the 5’ and/or 3’ strand of the hairpin arms (34). The pri-miRNA transcripts are converted into the hairpin precursor (pre-miRNAs) by cleavage of the flanking sequences by the DiGeorge syndrome Critical Region 8 (DGCR8)-Drosha complex. Translocation of the pre-miRNAs to the cytoplasm is mediated by Exportin-5 and followed by cleavage of the loop sequence by Transactivation Responsive RNA-Binding Protein (TRBP)-Dicer complex. One or both strands of the miRNA-duplex are incorporated into the Argonaute (Ago) containing RNA-induced silencing complex (RISC). The mature miRNAs direct the RISC complex to their target mRNAs. MiRNAs then influence the protein expressions either by blocking the translation into a protein or by degrading the mRNA (36).

Each miRNA regulates translation of multiple target genes, whereas one target transcript can be targeted by one or more miRNAs (37). It is estimated that over 60% of all protein-coding genes is regulated by miRNAs, which suggests a strict regulation of numerous processes and signalling pathways by these small non-coding RNAs (38).
Chapter 1

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Figure 2. MiRNA biogenesis (adapted from a figure made by N. Teteloshvili (39)). The miRNA genes are transcribed to primary miRNAs (pri-miRNAs) in the nucleus, by RNA polymerase II (RNA Pol II). The DiGeorge syndrome Critical Region 8 (DGCR8)-Drosha complex converts the pri-miRNAs into precursor miRNAs (pre-miRNAs), which are then translocated to the cytoplasm by exportin-5. Subsequently, the Transactivation Responsive RNA-Binding Protein (TRBP)-Dicer complex cleaves the pre-miRNAs into miRNA-duplexes. One strand of the miRNA-duplex is incorporated into the RNA-induced silencing complex (RISC) that contains the argonaute (Ago) proteins. The incorporated mature miRNAs direct the RISC complex to their target mRNAs. MiRNAs then influence the protein expressions either by blocking the translation into a protein or by degrading the mRNA.

Up to date, there are 2,588 human miRNAs according to the miRBase database (Release 21) (40). Several computational algorithms, including TargetScan, PicTar and miRanda, can predict the targets of these miRNAs (41). All programs have the disadvantage of not considering co-expression in the cell type of interest. Instead, the prediction programs are based on base pairing, thermodynamic stability of miRNA-mRNA interaction, conservation across species and the number of target sites per target transcript. Most algorithms integrate these features to increase the accuracy of miRNA target gene predictions. TargetScan, for example, predicts miRNA targets in vertebrates by combining seed region base pairing, thermodynamic stability of miRNA-mRNA interaction and conservation across species (42). One of the advantages of this prediction program is that it incorporates (more complete) the information about the number of isoforms of each target mRNA (43). The false positive rate is estimated to be 22-31% (41). Non-conserved targets are not predicted as miRNA targets when there is no perfect base pairing in the seed region, leading to false negatives (41). Overall, each prediction program has specific advantages and limitations.
Certainly, identification of miRNA targets using experimental approaches is preferred. Ago2-immunoprecipitation (Ago2-IP), either using unmodified cells or cells with modified levels of a specific miRNA, allows the capture of Ago2-miRNA-mRNA complexes. By performing mRNA microarray or RNA sequencing, the targets of the miRNAs can be identified. A popular variant of this approach is crosslinking immunoprecipitation followed by deep sequencing (Ago2-CLIP) (44). This technique uses ultraviolet irradiation to crosslink RNA to RNA-binding proteins before immunoprecipitation. Photoactivatable nucleosides can be used to improve the efficiency of crosslinking by ultraviolet radiation. In addition to these techniques, proteomics approaches such as stable isotope labelling with amino acids in cell culture (SILAC) are used to identify the targets of the miRNA of interest based changes at the protein level (45). Luciferase reporter assays are commonly used to investigate whether the mRNA of interest is a direct target of the miRNA. Alternatively, treatment of cells with miRNA mimics or inhibitors followed by Western blotting can confirm miRNA-dependent changes in protein expression levels.

Several miRNAs are found at different levels in either serum, plasma, whole blood, bronchoalveolar lavage, lung tissue, regulatory T cells or quadriceps of COPD patients in comparison to healthy individuals (46). However, limited studies are available investigating the role of miRNAs in lung tissue repair and remodelling. MiR-15b is expressed in fibrotic and emphysematous areas of the lung in COPD patients (47). Furthermore, miR-199a-5p promotes invasion, migration, proliferation and differentiation capacity of MRC-5 lung fibroblasts in vitro (48).

Numerous miRNAs are differentially expressed in different cell types upon ageing. In peripheral blood mononuclear cells from young and old individuals, 21 miRNAs have been found to be upregulated and 144 miRNAs downregulated with two fold in old individuals (49). Previous studies have suggested that miRNAs also regulate cellular senescence by targeting genes (e.g. p21 and SIRT1) involved in senescence-relevant pathways (50).

Several components of the TGF-β signalling pathway are directly or indirectly related to the regulatory pathway of a subset of miRNAs (31). SMAD proteins recognize pri-miRNAs with a conserved sequence (5’-CAGAC-3’) within the stem region and promote the conversion of those pri-miRNAs into pre-miRNAs (51, 52). In addition, TGF-β can indirectly regulate transcription of primary miRNA transcripts based on the SMAD-binding element in their promoters as shown for miR-27a in human lung fibroblasts (53) and let-7d in lung epithelial cells (54). Conversely, several miRNAs have been reported to influence TGF-β signalling by regulating the expression of TGF-β1, TGF-β receptors type I and type II, SMAD1-5 and SMAD7 (31, 47, 55). Altogether, these data strengthen the hypothesis that miRNAs contribute to the pathogenesis of COPD, at least in part via their effects on TGF-β-regulated tissue repair and remodelling.
Scope of the thesis

In this thesis, the aim was to identify miRNA expression changes in the lung. We studied the miRNA expression changes in primary lung fibroblasts that were affected by TGF-β stimulation and that were associated with COPD and current smoking. Furthermore, we aimed to identify gene and miRNA expression changes and their interactions related to ageing in bronchial biopsies from healthy individuals.

In chapter 2, the effect of TGF-β1 on the miRNA expression profile of primary lung fibroblasts and the role of TGF-β-regulated miRNAs was investigated. In addition, we identified the target genes of selected miRNAs using Ago2-RIP-Chip experiments. In chapter 3, small RNA sequencing was used to identify TGF-β1-regulated miRNAs in lung fibroblasts and to identify miRNAs that responded differently upon TGF-β1-stimulation in COPD lung fibroblasts compared to non-COPD control lung fibroblasts. Furthermore, the miRNA expression profiles of COPD and control lung fibroblasts were compared. Chapter 4 is focused on smoking-related miRNA expression changes in lung fibroblasts. In chapter 5, age-related gene and miRNA expression changes and their interactions were studied in bronchial biopsies from healthy subjects. The main findings in this thesis are summarized and discussed in chapter 6 and presented together with the future perspectives.
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General introduction & scope of the thesis


Chapter 2

Identification of transforming growth factor-beta-regulated microRNAs and the microRNA-targetomes in primary lung fibroblasts

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