CHAPTER 7

General discussion and perspectives
PRECISSION-CUT LUNG SLICES IN FIBROSIS RESEARCH

Idiopathic pulmonary fibrosis (IPF) is an incurable respiratory disease that is characterized by the pathological deposition of extracellular matrix (ECM) proteins in the lung interstitium [1]. Because approved treatments only slow the progression of IPF, there remains a great medical need for more effective and safer drugs. Unfortunately, the development of such drugs is challenging as the pathogenesis of IPF is not fully understood. To unravel the molecular mechanisms that drive the development of IPF and to lower the expression of fibrosis-related genes for therapeutic purposes, RNA interference (RNAi) can be exploited. RNAi is an endogenous mechanism that can be induced with small interfering RNA (siRNA) to achieve specific knockdown of messenger RNA (mRNA) and protein [2]. For that reason, siRNA is often used in vitro (cell cultures) and in vivo (animal studies). There is, however, a considerable gap between these models in terms of simplicity, flexibility, throughput, and translatability [3]. Appropriate experimental models are therefore required and they should recapitulate as many features of fibrosis as possible (e.g., cell-matrix interactions, cell heterogeneity, and immune responses).

Precision-cut lung slices are, accordingly, a useful model to fill this gap and can provide in-depth insights into fibrosis as slices retain structural and functional characteristics of the lungs [4]. Furthermore, the onset of wound-repair (fibrogenesis) can be studied in 'healthy' slices upon culture as a result of the slicing process and can be augmented with transforming growth factor β1 (TGFβ1), whereas the end-stage disease (fibrosis) can be assessed in slices prepared from leftover fibrotic tissue (i.e., surgical waste from IPF patients). Because lung slices can be prepared from either animal (e.g., guinea pigs, mice, non-human primates, pigs, rats, rabbits, or sheep) or human tissue, they also allow for the identification of potential species differences [3,5–11]. In addition, multiple slices with well-defined dimensions (i.e., the diameter, and thickness) can be quickly prepared from a single organ using specialized equipment, thereby increasing the number of experimental conditions that can be tested with a single animal. As a result, lung slices are well-suited not only to investigate fibrogenesis and fibrosis but also to contribute to the reduction, refinement, and replacement of animal experiments, while bridging the translational gap between in vitro and in vivo models as well as animal models and clinical studies.

As we wanted to study whether siRNA could attenuate fibrosis in lung slices, an appropriate transfection technique had to be selected because siRNA, which is negatively charged, cannot readily pass cell membranes [12]. Popular transfection techniques for in vitro experiments include the use of viral vectors, electroporation, and nanoparticles [13]. These techniques, however, are not suitable for transfecting slices. Even though viral vectors can enter cells in an efficient and evolutionary-optimized manner, they might also exert potent immunogenic effects, which are undesirable as inflammation influences fibrogenesis and fibrosis [14]. Although electroporation has been previously used to transfect brain slices, there are major concerns regarding its cytotoxic effects; because electroporation temporarily adjusts plasma membrane properties, other extracellular substances may also enter cells [15,16]. Using nanoparticles is also not recommended because they do not diffuse uniformly throughout the entire slice [17,18]. Electrostatic interactions between nanoparticles and outermost cells probably obstruct their diffusion into deeper tissue regions. Evidently, another technique had to be used to transfect lung slices.

To transfect lung slices without affecting their viability, we used self-deliverable (Accell) siRNAs. Accell siRNAs are duplex oligonucleotides that are chemically modified to facilitate internalization by cells and to circumvent degradation by nucleases. Though the exact modifications are unknown, inspection of the manufacturer’s patent portfolio revealed the internalization of Accell siRNA is probably facilitated by a lipid-conjugate, which can be either cholesterol, cholestanol, stigmasterol, cholanic acid, or ergosterol, that is attached to the sense strand of siRNA via an alkyl chain of 5 to 8 carbon atoms long [19]. Because of its increased lipophilicity, Accell siRNA is likely to enter cells via initial insertion of the lipid-conjugate into the plasma membrane followed by absorption of the siRNA molecule. We showed Accell siRNA uniformly transfected lung slices, thereby leading to specific and significant mRNA and protein knockdown. mRNA knockdown required 48 h of incubation, whereas protein knockdown required at least 96 h [3,20]. The time required for protein knockdown was substantial as the lifespan of slices was initially limited to 96 h. This means valuable experimentation time was restricted.

To address this limitation, we investigated whether the viability of lung slices could be improved by lowering the incubator oxygen concentration (from 80 to 20% O₂). In our study, we observed that lung slices cultured at 20% O₂ displayed fewer signs of cell death, anti-oxidant transcription, and acute inflammation as well as more cell proliferation. As the viability of lung slices was substantially improved by lowering the incubator oxygen concentration, we could also extend the incubation time from 96 to 144 h. Perhaps, the incubation time can be even further extended
by optimizing the culture medium composition (i.e., by adding growth factors) [21]. Although most studies describe culture times of a few days (24-96 h), some studies report culture times of a few weeks (table 1) [8,22–26]. However, it remains difficult to compare these studies as they analyzed different viability parameters, which are not always equally informative. To that end, complementary techniques should be used, such as a combination of biochemical and morphological analyses. Ultimately, the value of tissue slices comes from the fact that it represents a mini-model of an organ, so slices should retain their functional and structural features for as long as possible.

### TABLE 1. Overview of studies with precision-cut lung slices.

<table>
<thead>
<tr>
<th>Time</th>
<th>Species</th>
<th>Analytical technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>Mouse</td>
<td>LIVE/DEAD assay (esterase activity &amp; membrane permeability)</td>
<td>[22]</td>
</tr>
<tr>
<td>48 hours</td>
<td>Rat</td>
<td>Alamar Blue assay (oxidoreductase activity)</td>
<td>[8]</td>
</tr>
<tr>
<td>72 hours</td>
<td>Sheep</td>
<td>MTT assay (oxidoreductase activity)</td>
<td>[23]</td>
</tr>
<tr>
<td>96 hours</td>
<td>Pig</td>
<td>Light microscopy (bronchoconstriction &amp; ciliary activity)</td>
<td>[24]</td>
</tr>
<tr>
<td>1 week</td>
<td>Rat</td>
<td>H&amp;E staining (morphology)</td>
<td>[25]</td>
</tr>
<tr>
<td>2 weeks</td>
<td>Human</td>
<td>WST-1 assay (oxidoreductase activity) &amp; LIVE/DEAD assay</td>
<td>[26]</td>
</tr>
<tr>
<td>4 weeks</td>
<td>Sheep</td>
<td>LIVE/DEAD assay</td>
<td>[27]</td>
</tr>
</tbody>
</table>

### KNOCKDOWN OF HSP47 IN FIBROGENIC SLICES

After developing a transfection method for lung slices, we evaluated the therapeutic effect and mechanism of Serpinh1-targeting siRNA to treat fibrosis. Serpinh1 was selected as it encodes heat shock protein 47 (HSP47), which is a molecular chaperone that resides in the endoplasmic reticulum (ER) and it is essential for the folding and intracellular trafficking of procollagen molecules [28,29]. Targeting HSP47 instead of ECM proteins, such as collagen type 1 (COL1), has an advantage because, upon knockdown, myofibroblasts become apoptotic due to sustained ER stress, which is caused by the accumulation and aggregation of misfolded procollagen molecules [30]. Knockdown of HSP47 is therefore hypothesized to not only halt progression of fibrosis but also alleviate existing fibrosis as myofibroblasts may disappear from fibrotic lesions. So far, encouraging results have been obtained as described by several publications which demonstrated Serpinh1-targeting siRNA alleviated pulmonary, hepatic, renal, peritoneal, dermal, and vocal fold fibrosis in animal models [31–36]. Furthermore, Nitto Denko Corporation (Osaka, Japan) and Bristol-Myers Squibb (New York, United States) are currently conducting clinical studies with vitamin A coupled liposomes that contain Serpinh1-targeting siRNA (ND-L02-s0201/BMS-986263) (table 2). The sponsors of these studies postulate that coating liposomes with vitamin A improves their uptake by myofibroblasts. Therefore, HSP47 appears to be a promising therapeutic target for knockdown by siRNA to treat fibrotic diseases.

### TABLE 2. Overview of clinical studies with ND-L02-s0201/BMS-986263.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Phase</th>
<th>Status</th>
<th>Time</th>
<th>Size</th>
<th>Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT01858935</td>
<td>1</td>
<td>Completed</td>
<td>05/2013 → 02/2014</td>
<td>56</td>
<td>Healthy volunteers</td>
</tr>
<tr>
<td>NCT03241264</td>
<td>1</td>
<td>Completed</td>
<td>08/2016 → 10/2016</td>
<td>12</td>
<td>Healthy volunteers</td>
</tr>
<tr>
<td>NCT02227459</td>
<td>1b/2</td>
<td>Completed</td>
<td>10/2014 → 05/2016</td>
<td>25</td>
<td>Patients with hepatic fibrosis</td>
</tr>
<tr>
<td>NCT03538301</td>
<td>2</td>
<td>Recruiting</td>
<td>06/2018 → 03/2020</td>
<td>120</td>
<td>Patients with IPF</td>
</tr>
<tr>
<td>NCT03420768</td>
<td>2</td>
<td>Recruiting</td>
<td>02/2018 → 07/2020</td>
<td>165</td>
<td>Patients with hepatic fibrosis</td>
</tr>
</tbody>
</table>

To characterize the therapeutic effects of Serpinh1-targeting siRNA for treating fibrosis, we treated lung slices with TGFβ1 and Serpinh1-targeting Accell siRNA. After 144 h of incubation, multiple aspects of fibrogenesis were analyzed, such as mRNA expression of fibrogenesis-related genes, fibronectin secretion into culture medium, and expression of alpha smooth muscle actin (α-SMA) as well as collagen secretion and its deposition into the ECM. Although robust and specific knockdown of HSP47 (~90%) was achieved, we did not observe diminished collagen secretion or deposition. This finding contradicts previous research where HSP47 knockdown was found to suppress collagen secretion by fibroblasts [36]. It cannot be ruled out, however, that residual HSP47 (~10%) in lung slices was sufficient for the secretion of procollagen molecules. Apoptosis of myofibroblasts is also unlikely to have occurred as mRNA expression of fibrogenesis-related genes and expression of α-SMA remained stable upon knockdown of HSP47. Perhaps, misfolded and aggregated procollagen molecules in the ER were degraded through autophagy, which could have prevented ER stress and subsequent apoptosis [37]. Nevertheless, many questions regarding the role of HSP47 in fibrosis remain unanswered.
As reported previously, six animal studies clearly showed therapeutic effects of Serpinh1-targeting siRNA after 3 to 4 weeks of frequent administration [31–36]. Unfortunately, those studies did not provide data with respect to the effects over time. Consequently, no insights into the exact therapeutic mechanism was obtained. Their findings would have been even more interesting if they also monitored collagen secretion/synthesis and deposition over time. It is possible that knockdown of HSP47 only affected collagen folding but not its secretion, thereby leading to the gradual incorporation of misfolded collagens into ECM which could have made the ECM more vulnerable to degradation by matrix metalloproteinases. Additionally, apoptosis of myofibroblasts might have been prevented by rapid elimination of misfolded and aggregated procollagen molecules through autophagy. To determine whether autophagy played a role, it would be interesting to repeat the experiments and include the use of an autophagy inhibitor. More research on the therapeutic effects of Serpinh1-targeting siRNA is therefore greatly desired.

The emergence of new analytical techniques could help us to study the therapeutic potential of Serpinh1-targeting siRNA. Transcriptome profiling, for example, has recently been used to rapidly characterize the complete set of RNA transcripts in lung slices [38]. Nevertheless, this technique is usually applied to analyze whole tissue and does not reveal phenotypes of individual cells or cell types. Characterizing phenotypes of individual cells (or cell types) in a heterogeneous multicellular environment would greatly advance research as it avoids masking effects caused by the inability to detect relevant changes in non-abundant cells. To that end, laser-capture microdissection or fluorescence-activated cell sorting followed by single-cell transcriptome profiling could be used to detect altered gene expression in specific structures (e.g., airways) or cells (e.g., myofibroblasts), respectively [39]. Extensive characterization of the extracellular matrix (ECM), which comprises ~300 different types of proteins, also deserves more attention because immunohistochemical stainings would be impractical to use [40,41]. Stable isotopomer labeling and liquid chromatography/mass spectrometry based isotopomer analysis, for example, could offer insights into the composition of the ECM and the synthesis rate of its constituents [42].

**DELIVERY OF SIRNA TO MYOFIBROBLASTS**

In case knockdown of HSP47 turns out to be a successful approach to treat IPF, steps can be made towards the development of a dosage form for Serpinh1-targeting siRNA. Parenteral administration of siRNA is not preferred as siRNA is rapidly excreted via the kidneys and degraded by nucleases in the serum [2]. To avoid these issues and to achieve site-specific delivery of siRNA in the lungs, pulmonary administration would be an attractive option. Before selecting an inhalation device and developing an inhalable formulation, strategies should be identified to ensure siRNA is delivered to the target cells (myofibroblasts). To that end, siRNA has to cross several biological barriers. Upon inhalation, siRNA is first dissolved in mucus of the airways and alveolar lining fluid [43]. These fluids reduce direct contact between siRNA and cells. While dissolved in mucus and alveolar fluid, siRNA needs to avoid mucociliary clearance and macrophage-mediated phagocytosis [44]. To reach myofibroblasts, siRNA molecules should subsequently cross the respiratory/alveolar epithelium, after which they need to diffuse through the ECM [45]. In IPF patients, this ECM is abundant and tightly cross-linked, thereby limiting the diffusion of large molecules. Reaching myofibroblasts, however, it not sufficient to achieve knockdown as siRNA is negatively charged and does not easily enter cells.

Though some animal studies reported knockdown can be achieved with naked siRNA, it might be necessary to use delivery vectors to overcome these barriers [46,47]. In recent years, considerable progress has been made regarding the development of delivery vectors for siRNA, including but not limited to nanoparticles and bioconjugates [48,49]. The use of nanoparticles, however, is not recommended for siRNA delivery to myofibroblasts. The main issue with nanoparticles is their extremely limited diffusion through (fibrotic) ECM. In fact, nanoparticles larger than 60 nm, cannot diffuse through dense, collagen-rich, ECM at all [50]. Alternatively, siRNA can be conjugated with cell-penetrating peptides (e.g., transportan and penetratin) or lipid molecules (e.g., sterols and fatty acids) to enable delivery to myofibroblasts [48,51]. siRNA bioconjugates are smaller than traditional nanoparticles (7.5 nm vs. 100-200 nm) and are expected to have the ability to pass through the alveolar epithelium and ECM [52]. It is also strongly recommended to further modify the chemical structure of siRNA to make it more resistant to degradation by nucleases. Sugars, backbones, and bases, for example, can be chemically modified without affecting the biological activity of siRNA [53].

Aside from being effective in the desired target cell, myofibroblasts, it is also important to evaluate the safety, stability, and commercial viability of siRNA bioconjugates. For instance, siRNA bioconjugates should have a limited toxicity and immunogenicity. Because siRNA introduced into the body can potentially activate the innate immune system via pattern recognition receptors (PRRs), care should be taken to minimize off-target effects by optimizing the siRNA sequence and by chemically modifying selected sequences to circumvent activation of PRRs.
Currently, it is not clear which particle preparation technique is preferred for more expensive, considerably slower, and prone to batch-to-batch variation [56]. An alternative, spray-freeze drying can be used for such drugs, despite being has the potential to affect the stability of temperature-sensitive drugs [57]. As known for its robustness, high throughput, and scalability, but the heat exposure obviously, each technique has its strengths and weaknesses. Spray drying is known for its robustness, high throughput, and scalability, but the heat exposure has the potential to affect the stability of temperature-sensitive drugs [57]. As an alternative, spray-freeze drying can be used for such drugs, despite being more expensive, considerably slower, and prone to batch-to-batch variation [56]. Currently, it is not clear which particle preparation technique is preferred for

**TOWARDS PULMONARY ADMINISTRATION OF siRNA**

Pulmonary administration has been proposed as a strategy to achieve local delivery of siRNA [49]. This requires the selection of an appropriate inhalation device, such as a nebulizer, soft mist inhaler, metered-dose inhaler, or dry-powder inhaler (DPI) [2]. Among these options, DPIs are preferred for the delivery of siRNA, which is much more stable in a dry state than in a liquid formulation [55]. Nonetheless, it is not known if DPIs are suitable for patients suffering from IPF. The delivery of a dry powder to fibrotic lesions, for instance, could be impaired due to distortions in the tissue architecture. There are some indications, however, that DPIs can be used to treat IPF patients. For example, after successfully completing a phase 1b/2a trial with patients suffering from IPF (NCT02257177), Galecto Biotech (Copenhagen, Denmark) has started a phase 2b trial (NCT03832946) to investigate the efficacy and safety of galectin-3 inhibitor TD139, which is to be taken twice-daily with a DPI. Results from their phase 1b/2a trial have not been published yet, but the initiation of a phase 2b trial suggests encouraging results were obtained.

If the use of DPIs is well-tolerated in IPF patients, the preparation and formulation of siRNA-containing dry powders can be considered. Spray or spray-freeze drying can be used to prepare dry powders with an aerodynamic size distribution (1-3 μm) that allows for particle deposition in the entire lung, including the alveolar region [56]. Both particle engineering techniques are based on the atomization of a drug solution or suspension into small droplets, which are either dried due to solvent evaporation (spray drying) or sublimation (spray-freeze drying) [55]. Obviously, each technique has its strengths and weaknesses. Spray drying is known for its robustness, high throughput, and scalability, but the heat exposure has the potential to affect the stability of temperature-sensitive drugs [57]. As an alternative, spray-freeze drying can be used for such drugs, despite being more expensive, considerably slower, and prone to batch-to-batch variation [56]. Currently, it is not clear which particle preparation technique is preferred for

siRNA. Some studies have demonstrated that both techniques result in siRNA-containing dry powders with good aerodynamic properties [58,59].

Regardless of the powder preparation technique, excipients should be carefully chosen as they directly affect the overall performance of DPIs. Because of its potency, siRNA is likely to be administered at a relatively low dose [55]. This means a bulking agent (e.g., sugars or polyols) is required to convey the siRNA. Nevertheless, not all bulking agents are equally appropriate. To deliver siRNA into fibrotic lungs, it would be preferred to use quickly-dissolving and non-reducing bulking agents with good dispersion properties, such as inert (poly-) saccharides [60]. It might also be necessary to add a dispersion enhancer, which can be used to lower cohesive forces between powder particles. Leucine, for example, is a dispersion enhancer that can be used to prepare powder particles with a corrugated surface, thereby improving dispersibility [61]. The tolerability of excipients, however, should not be ignored. Mannitol, for example, has been previously used to prepare inhalable siRNA-containing dry powders, though it is also used in bronchial provocation challenges to induce an asthmatic response [58,60,62,63]. As a result, mannitol is probably not suitable for IPF patients because it may lead to coughing, airway irritation, and bronchospasms [63].

**CONCLUSION**

In this thesis, we set out to explore the use of siRNA within the context of pulmonary fibrosis. On the basis of published animal studies, we first evaluated whether pulmonary administration can be used to deliver siRNA into the lungs (**chapter 2**). Although this approach is promising, several problems were identified, such as the lacking translatability between *in vitro* and *in vivo* models. To address this issue, we developed and optimized a transfection method for lung slices to induce not only mRNA knockdown but also protein knockdown (**chapter 3** and **4**). Before the transfection method was applied to investigate the effects of siRNA, we performed an extensive optimization with respect to the viability of lung slices (**chapter 5**). Thereafter, we assessed the therapeutic potential of *Serpins* -targeting siRNA in lung slices that displayed a fibrogenic phenotype (**chapter 6**). Although we did not observe diminished collagen secretion and deposition in slices upon knockdown of HSP47, we strongly believe future studies should be conducted to elucidate the role of HSP47 in fibrosis as therapeutic effects observed *in vitro* (cell cultures) may not be entirely representative of what actually happened *in vivo* (animal studies). We hope that our research will serve as a basis for such studies.
BIBLIOGRAPHY


### Deletion of the Collagen-specific Molecular Chaperone Hsp47

### Development and preclinical efficacy of novel approaches to target the collagen network in fibrosis.

1. **Protein instability and immunogenicity:** Roadblocks to clinical translation.

2. **Laser microdissection of the alveolar duct enables single-cell analysis:** of interstitial fibroblasts in lung fibrosis.

3. **Using two-fluid nozzle for spray freeze drying:** to produce porous silica-based nanoparticles for treating fibrosis.

4. **Proteomic Analysis of Altered Extracellular Matrix Turnover in Fibrotic Lungs:** using mass spectrometry.

5. **Chapter 7:** Focus on the role of Hsp47 in fibrotic processes.

### Key References


