Modeling pulmonary fibrosis by AAV-mediated TGFβ1 expression – a proof of concept study for AAV-based disease modeling and riboswitch-controlled vector production

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I Summary

Adeno-associated virus (AAV) vectors have gained considerable attention as tools for the genetic manipulation of various cell types, tissues and organs \textit{in vitro} and \textit{in vivo}, which is facilitated by their non-pathogenicity, weak immunogenicity, low biosafety requirements (biosafety level S1) and comparatively easy recombinant production. However, despite the identification of AAV6.2, a capsid protein VP1 F129L point-mutated variant of AAV6, which showed superior transduction efficiency to other serotypes in the lung of mice, no studies have utilized AAVs for pulmonary disease modeling so far. To investigate AAV6.2's suitability for this purpose, first, its pulmonary transduction efficiency and cellular tropism, transgene expression stability and immunogenicity were studied. Our results demonstrated that AAV6.2 enables broad transduction of the murine lung with stable transgene expression observed in bronchial epithelial and type II alveolar epithelial cells over the full tested time period of four months. Notably, in contrast to a commonly used Adenovirus-5 vector, AAV6.2 did not induce any measureable acute inflammation upon intratracheal administration, thereby lowering the risk of altering relevant readouts by vector-mediated immune responses. Moreover, AAV6.2-mediated overexpression of TGFβ1 dose-dependently induced pulmonary fibrosis in mice, which mirrored several key features of the human pathology, thereby establishing proof-of-concept for AAV-mediated disease modeling. A time course experiment in direct comparison to Bleomycin-induced lung fibrosis – the most widely used model of pulmonary fibrosis – identified key differences and commonalities in disease onset and progression. We found that the most distinct difference between both models lays in an acute injury/inflammation response in the Bleomycin model prior to fibrosis development, as opposed to a phase of relatively simultaneously occurring fibrosis and inflammation in the AAV model. By next generation sequencing, mRNA and microRNA expression changes were tracked and used to identify common disease signatures during fibrosis onset and maintenance. Utilizing this approach and correlation computation using lung functional changes, protein and miRNA candidates were identified, which might present attractive candidates for fibrosis drug discovery research, among them lysyl oxidase-like 2 (LOXL2), hyaluronan synthase 2 (HAS2), fibromodulin (FMOD) as well as miR-181a-5p, miR-676-3p and miR-192-5p. Furthermore, suitability of the AAV6.2-TGFβ1 model for compound testing was also established by pharmacological intervention using a type I TGFβ receptor inhibitor. Finally, to facilitate production of AAV vectors independent of the transgene used, we developed a novel gene regulation system based on an AAV-integrated artificial, self-cleaving, guanine-responsive riboswitch. This approach enabled efficient production of high-titer AAV vectors with close-to-normal \textit{in vivo} bioactivity, and further enabled dynamic transgene expression control after AAV transduction \textit{in vitro}. Following further engineering, such vectors might be ultimately used as gene expression control devices in AAV gene therapy.
Zusammenfassung

1 Introduction

Modeling pulmonary fibrosis by AAV-mediated TGFβ1 expression – a proof of concept study for AAV-based disease modeling and riboswitch-controlled vector production
1.1 Adeno-associated virus (AAV) vectors

1.1.1 AAV biology

Exactly fifty years ago, Adeno-associated viruses (AAV) were first discovered as a contamination of a simian adenovirus preparation in a laboratory at University of Pittsburgh (1). Already in this first description, it was realized that replication of AAV can only occur in presence of Adenovirus and, as found out later, Herpes simplex virus (2). Due to these findings, AAVs, which are small, non-enveloped viruses, are ascribed to the genus of Dependoviruses, which belong to the family of Parvoviridae. Besides eleven different AAV serotypes (3), over 300 AAV variants have been isolated to date and examination of human blood samples demonstrated that AAV infections are commonly encountered and widely distributed. However, despite a high serum prevalence of anti-AAV antibodies (4)(5), which is dependent on the AAV serotype examined (AAV2 (72 %) > AAV1 (67 %) > AAV9 (47 %) > AAV6 (46 %) > AAV5 (40 %) > AAV8 (38 %)), no disease has been found to be associated with AAV, underscoring the accepted view of AAV being non-pathogenic. AAVs harbor a single-stranded DNA genome flanked by palindromic inverted terminal repeats (ITR) that is packaged into an icosahedral capsid of approximately 20 nm in diameter. The wild type (wt) AAV genome comprises about 4.7 kb, can be either positive- or negative-sensed and harbors three open reading frames (ORF) within the rep (replication) and cap (capsid) genes (Figure 1a).

![AAV structure](image)

Figure 1: Genomic and capsid structure of Adeno-associated virus

(a) The AAV genome contains two genes, rep and cap, which harbor three ORFs flanked by inverted terminal repeats (ITR). The rep ORF encodes the four non-structural proteins Rep78, Rep68, Rep52 and Rep40, which result from alternative splicing. The cap ORF encodes the three capsid proteins VP1-3 and the assembly-activating protein (AAP) that is expressed from an alternative ORF. Genetic regions encoding amino acids exposed on the capsid surface are marked by black lines within cap. (b) Crystal structure of the AAV2 capsid (6) with colored hypervariable regions matching the corresponding color-coded genetic regions (arrows) in the cap gene shown in (a). Figure reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, Kotterman and Schaffer DV, Engineering adeno-associated viruses for clinical gene therapy. (7), copyright 2014.
Introduction

Two overlapping, intron-containing mRNA transcripts are expressed from the p5 and p19 promoters contained in the rep ORF, therefore encoding four proteins, resulting from optional splicing events: Rep78, Rep68, Rep52 and Rep40. All Rep proteins share helicase and ATPase activity, while Rep78 and Rep68 additionally have sequence-specific endonuclease activity (8)(9). The cap gene encodes the three capsid proteins VP1 (87 kDa), VP2 (72 kDa) and VP3 (62 kDa), which self-assemble in a ratio of 1:1:10 to form the AAV capsid (whose crystal structure is shown in Figure 1b), thereby incorporating 60 VP subunits. The three capsid proteins are expressed from a common promoter, termed p40, and result from mRNA splicing events (10)(11)(12); therefore, VP3 and VP2 represent N-terminally differentiated versions of VP1. From an alternative ORF within the cap gene, the assembly-activating protein (AAP) is expressed, which targets the capsid proteins to the nucleolus, where it promotes capsid formation (13).

Wild type AAV’s life cycle can either be lytic or lysogenic. Upon cell attachment by binding specific primary and co-receptors, which depends on the individual capsid of an AAV serotype/variant (see Table 1 for AAV serotype-dependent receptor usage), the virus gets endocytosed in clathrin-coated pits, from where it escapes following acidification of the endosome (14)(15). The exact mechanism of the subsequent nuclear entry is still not fully understood, however, recent research suggests that AAV uses the classical nuclear import pathway via nuclear pore complexes to access the nucleus (16). Depending on whether or not co-infection with a helper virus occurs, AAV either pursues a lytic or lysogenic pathway. In absence of a helper virus, wild type AAV can integrate into a specific region within human chromosome 19, termed AAVS1 (17)(18), which represents the main spot (45 % of all events) of AAV integration (19). AAV integration was shown to be dependent on the presence of Rep78 or Rep68 in trans and on cis-acting, Rep-binding element (RBE)-containing DNA structures as present within the ITRs or the viral p5 promoter (20)(21)(22). Upon co-infection with a helper virus, the AAV genome can be rescued by Rep-mediated excision, replication and packaging into the capsid. In general, the replication of AAV is believed to occur according to following model (23)(24): The free 3’-hydroxyl group at the ITR hairpin serves as a self-primer for the attachment of DNA polymerase and initiation of replication. Following the synthesis of the second strand, the duplex molecule is cut by Rep78 and Rep68, which specifically bind the RBE and, due to their endonuclease activity, generate a nick at the trs, thereby forming a new 3’-hydroxyl group that DNA polymerases can use as a starting point for copying the ITR. After the renaturation of the palindromic terminal sequences, the newly formed ITR provides a new starting point for single-strand displacement, synthesis of a novel second strand and generation of a duplex molecule, thereby restarting the genome replication circle. Studies also suggest that Rep52 and Rep40 are involved in the generation and accumulation of single-stranded genomes from the duplex molecules temporarily synthesized during genome replication (25). Moreover, it
has been proposed that they facilitate encapsidation of the ssDNA AAV genome by unwinding and transferring the DNA into pre-formed AAV capsids (26)(27), which are assembled in the nucleolus before being transported into the nucleoplasm for the formation of intact AAV particles (28).

Table 1: Receptors and preferred tissue tropism of different AAV serotypes/variants

<table>
<thead>
<tr>
<th>AAV</th>
<th>Glycan receptor</th>
<th>Co-receptor/other</th>
<th>Tissue tropism</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV1</td>
<td>N-linked sialic acid</td>
<td>Unknown</td>
<td>SM, CNS, retina, pancreas</td>
</tr>
<tr>
<td>AAV2</td>
<td>HSPG</td>
<td>FGFR1, HGFR, LamR, CD9 Tetranspanin</td>
<td>VSMC, SM, CNS, liver, kidney</td>
</tr>
<tr>
<td>AAV3</td>
<td>HSPG</td>
<td>FGFR1, HGFR, LamR</td>
<td>Hepatocarcinoma, SM</td>
</tr>
<tr>
<td>AAV4</td>
<td>O-linked sialic acid</td>
<td>Unknown</td>
<td>CNS, retina</td>
</tr>
<tr>
<td>AAV5</td>
<td>N-linked sialic acid</td>
<td>PDGFR</td>
<td>SM, CNS, lung, retina</td>
</tr>
<tr>
<td>AAV6</td>
<td>N-linked sialic acid, HSPG</td>
<td>EGFR</td>
<td>SM, SM (i.v.), heart, lung</td>
</tr>
<tr>
<td>AAV7</td>
<td>Unknown</td>
<td>Unknown</td>
<td>SM, retina, CNS</td>
</tr>
<tr>
<td>AAV8</td>
<td>Unknown</td>
<td>LamR</td>
<td>Liver, SM, CNS, retina, pancreas, heart</td>
</tr>
<tr>
<td>AAV9</td>
<td>N-linked galactose</td>
<td>LamR</td>
<td>Liver, heart (i.v.), brain (i.v.), SM (i.v.), lungs, pancreas, kidney (i.v.)</td>
</tr>
</tbody>
</table>

Table 1 shows the primary glycan and co-receptors for AAV1-9 and their preferred tissue tropism upon local administration or as indicated. CNS, central nervous system; EGFR, epidermal growth factor receptor; FGFR1, fibroblast growth factor receptor 1; HGFR, hepatocyte growth factor receptor; HSPG, heparan sulfate proteoglycan; i.v., intravenous; LamR, laminin receptor; PDGFR, platelet-derived growth factor receptor; SM, skeletal muscle; VSMC, vascular smooth muscle cell. Table adapted from Nonnenmacher M and Weber T, Gene Ther 2012 (29). References for tissue tropism are depicted in the original publication.

1.1.2 Generation of recombinant AAV vectors

Based on the fact that the ITR sequences harbor all cis-acting elements required for the replication and packaging of the AAV genome, recombinant AAV vectors can be generated by completely replacing the virus encoding sequence between the ITRs with a transgene cassette of interest, and providing the sequences encoding replication and capsid proteins in trans (Figure 2). More specifically, for recombinant AAV production, most approaches exploit plasmids harboring AAV2 ITR sequences flanking a transgene expression cassette consisting of a promoter, a transgene of interest and a poly(A) signal and one or two additional plasmids containing the rep and cap genes of the desired serotype along with the required adenoviral helper genes E1, E2a, E4ORF6 and VA (30)(31)(32). Most commonly, HEK-293 cells are transiently co-transfected with these plasmids and grown for two to five days before being lysed for the isolation of AAV particles and subsequent purification. As HEK-293 cells express E1a and E1b, in this case transfection with E2a, E4ORF6 and VA is sufficient. Due to this helper virus-free production strategy, the only
remaining viral elements in recombinant AAVs are the ITRs, which is why AAV vectors are replication-deficient and chromosomal integration is a highly unlikely event. For transgene expression, the host cell transforms the single-stranded AAV genome (transgene cassette) into a transcriptionally amenable double-stranded DNA, which subsequently gets processed to mRNA. However, double strand synthesis is believed to be a major limiting step in AAV transduction (33)(34), which is why efforts were undertaken to bypass it. It was found that by mutating the terminal resolution site in one of the ITRs, genome cleavage was prevented, resulting in a vector genome with complementary strands that backfold to immediately become a double-stranded template for transcription (35)(36). Such self-complementary AAVs (scAAV) were shown to induce a much faster onset and also higher levels of transgene expression and have become a routinely used vector tool in numerous studies for both, research and clinical applications (37).

Figure 2: Generation of recombinant Adeno-associated virus vectors

Recombinant AAV vectors are generated by inserting a transgene expression cassette between the AAV inverted terminal repeats, thereby replacing the AAV rep and cap genes (also see Figure 1). AAV rep and cap as well as required adenoviral helper genes are provided *in trans* on a separately co-transfected plasmid. Upon transfection of producer cells (e.g. HEK-293), the AAV capsid is assembled from VP1, VP2 and VP3 aided by the assembly-activating protein (AAP), and Rep proteins guide the ITR-flanked transgene cassette into the pre-assembled capsid. Figure reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, Kotterman and Schaffer DV, Engineering adeno-associated viruses for clinical gene therapy. (7), copyright 2014.

Because transient transfection of adherently grown cells like HEK-293 is difficult to scale up, several attempts have been pursued to establish alternative AAV production systems. A common strategy is the generation of packaging cell lines that stably express AAV rep and cap genes, however, their generation is complicated by the finding that Rep expression suppresses cell
growth (38), therefore requiring dynamic expression control using inducible systems (39). Moreover, flexible production of different AAV variants is hardly feasible using stable cell lines, since an individual cell line for each AAV capsid variant would be needed. An established alternative production platform relies on SF9 insect cells that can be grown in suspension, which are infected with a baculovirus carrying the required AAV transgene, AAV rep/cap and adenoviral helper genes (40). Additionally, a platform using stably rep/cap-expressing SF9 cells has been established for AAV1-12 (41). However, given that the generation of baculoviruses is relatively time-consuming, HEK-293 cell-based AAV production remains the most commonly used protocol in pre-clinical research to date.

Despite the realization that AAV vectors are also found in the cell supernatant, which seems to depend on both the AAV variant produced and the time that the producer cells are kept in culture after transfection (42), AAVs are mostly purified from the cell lysate of transfected cells, which is usually obtained by three consecutive freeze and thaw cycles. Early purification protocols relied on isopycnic cesium chloride (CsCl)-based ultracentrifugation as the core purification step (43) and further optimization included centrifugation series using up to three of these gradients (44) as well as incorporation of PEG precipitation steps (45). Later on, iodixanol, a clinically used contrast agent was found to be a suitable medium for the preparation of density step gradients and more rapid purification of AAV (46)(47). Given that both, CsCl- and iodixanol-based protocols have advantages (faster process and slightly higher purity using iodixanol, better full-to-empty AAV particle ratio using CsCl) and disadvantages (cytotoxicity of CsCl, therefore requiring dialysis of the AAV preparations prior to use) (48), both protocols are widely established and probably equally commonly used for the preparation of AAV vectors as research tools.

Nevertheless, to enable large scale purification of AAV vectors, alternative, scalable protocols for the efficient preparation of highly pure vector stocks are desired. To this end, methods making use of the physicochemical properties of AAV along with the increasing knowledge on receptor binding and protein-interactions (also see Table 1), such as two-phase partitioning (49), affinity- and ion-exchange chromatography, have been established. Reported affinity-based protocols rely on heparin-binding by AAV2 (50), mucin-binding of AAV5 (sialic acid is a component of mucin) (51) and most recently, specific AAV capsid capturing by a commercially available single-chain antibody affinity medium (52)(53). Ion-exchange protocols for AAV1, 2, 4, 5 and 8 mostly exploit the anionic character of the AAV capsid (54)(55)(56)(57)(58)(59)(60), although cation exchange was recently demonstrated based on the finding that the AAV capsid gets protonated at low pH (61). However, some serotypes such as AAV9 seem to be largely refractory to conventional column purification and hence call for further optimized purification strategies (62)(61). Moreover,
Introduction

Besides the difficulty that most of these protocols are not universally applicable for several AAV capsid variants and therefore require the development of individual serotype-dependent protocols, one of the main challenges remains the efficient separation of full (intact) and empty AAV particles. Given the importance of minimizing the amount of empty particles with regard to receptor occupation and potential immunogenicity, particularly for clinical vector applications, empty particle depletion is a crucial requirement, which so far can only be accomplished by density ultracentrifugation.

1.1.3 AAV capsid engineering for preclinical and clinical applications

Besides the optimization of vector production and purification, engineering efforts also focus on the generation of novel AAV capsids with altered tropism, enhanced transduction efficiency or reduced immunogenicity (63)(7)(64). While such engineering efforts extend the applicability of AAV as a research tool, the long-term goal mostly lies in improving applicability of AAV vectors as vehicles for gene therapy. AAV’s property to be non-pathogenic along with the fact that it transduces both dividing and non-dividing cells render it highly attractive for this purpose; yet, despite some successes, for example for the treatment of Leber’s congenital amaurosis (AAV2) (65)(66) and Lipoprotein lipase deficiency (AAV1) (67), other clinical studies clearly demonstrated the problem of pre-existing and acutely induced neutralizing antibodies as well as capsid-directed T-cell responses, which limit long-term gene expression in non-immune privileged tissues (68)(69)(70). Furthermore, by altering the tropism of AAV variants, transduction of so far non-targetable tissues and cells, such as the kidney, pancreas (β-islets), monocytes and T-cells might become feasible in the future.

Several strategies have hence been pursued to alter the AAV capsid, including the insertion/substitution of peptides (71)(72)(73) or protein ligands (74)(75) into exposed surface loops of the AAV capsid, the random combination of capsid proteins (“mosaic viruses”) (76), the combination of capsid domains or amino acids of different AAV variants (“chimeric viruses”) (77), the random shuffling of capsid sequences by error-prone PCR, DNA-shuffling (78)(79)(80) and the combination of several of the aforementioned approaches.

One example for a rationally designed AAV variant is AAV6.2, which was found to show exceptional transduction efficiency of both, human airway epithelial cells in vitro and the murine lung in vivo (81). In this study, superior transduction efficiency was demonstrated in comparison to AAV1, 2, 5, 6, 7, 8 and 9 as well as 19 additional, novel AAV isolates. AAV6.2 is based on AAV6 but carries a single point mutation, resulting in a phenylalanine-leucine substitution at position 129 (F129L) in the VP1 protein. This mutation was inserted based on the fact that all other
primate AAVs apart from AAV5 have a leucine at this position, whereas AAV6 has a phenylalanine (82). This singleton residue also represents one of the six amino acids that distinguish AAV6 from AAV1 (83). Notably, the moderate heparin binding ability of AAV6 (which is absent in AAV1) is not altered by the F129L mutation, but likely depends on a second singleton, K531E (83). Interestingly, residue 129 lies in a PLA2 domain within VP1 that is predicted to fold into the inner part of the assembled capsid but becomes externalized during endocytic trafficking (84)(85). It is therefore speculated that the F129L mutation might facilitate endosomal escape, rather than altering receptor-mediated transduction.

1.1.4 Applications of AAV vectors in respiratory research

Apart from AAV6.2, several other naturally occurring AAV variants have proven their ability to transduce lung tissue in mice. Following intranasal or intratracheal application, most studies report the highest overall lung transduction efficiency using AAV5 and AAV6, followed by AAV1 and AAV9 (86)(87)(88)(81). However, AAV9 has a specific tropism for alveolar cells but does not transduce bronchial epithelial cells. Therefore, when focusing on alveolar epithelium, AAV9 was similarly efficient as AAV5 and AAV6 and more efficient than AAV1 (89)(81). Contrary to the observations of Limberis and colleagues, one study reported most efficient lung transduction using AAV8 (90). When investigating the tissue tropism following intravenous administration, Zincarelli and colleagues found efficient expression in the upper thorax using AAV4, 6 and 9 followed by AAV7 and AAV8 (91). However, significant differences in overall tropism were observed: While AAV4 induced specific expression in the heart and the lung, AAV1 and 6 mainly showed expression in the lung, heart and hindlimb, whereas AAV7 and AAV9 demonstrated a more uniform transduction pattern throughout the body. Despite the specific and efficient transduction of the lung using AAV4, however, no studies examining intratracheal delivery using this vector have been reported so far. Further studies could help unravel whether transduction occurs mainly in the endothelium or whether AAV4 might transcytose into the lung parenchyma.

Following the realization that AAV tropism in mice does not always translate to primates including humans, several studies also used differentiated human airway epithelial cells to increase translatability. In these studies, AAV6.2 was found to be most efficient for apical transduction, followed by its parental strain AAV6 as well as AAV1, AAV9 and AAV5 (81)(86)(88). In addition, several other mutants also demonstrated efficient transduction, as did a variant called AAV2.5T, which was identified by directed evolution using a PCR- and in vitro-recombination-based chimeric capsid approach (92)(93).
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During the last twenty years, a plethora of studies have exploited AAV vectors as research tools to overexpress cDNAs, siRNAs and miRNAs, including Cre recombinase and CRISPR/Cas9 for the modulation and characterization of gene function in hard-to-transfect cell lines and in vivo model organisms. However, as also evident from the number of articles resulting from pubmed abstract searches using the terms “Adeno-associated” in conjunction with either “intratracheal OR intranasal” (54 hits), “intrathecal OR intracranial OR stereotactic” (132), “intravenous” (307), “intramuscular (329)” or “intravitreal (102)”, AAV studies in the lung are generally comparatively sparse. In fact, most of the AAV-based studies in the context of respiratory research focused on the evaluation of AAV tropism and transduction efficiency along with vaccination studies, where nasal AAV-mediated antigen expression was explored (94)(95). In contrast, only a handful of studies exploited AAVs to investigate pulmonary gene function upon overexpression (96)(97)(98)(99)(100), and studies towards AAV-mediated transgenic disease modeling are nonexistent. Given that adenoviral gene transfer to the lung, which has been most commonly used until now (101), is associated with inflammation and cellular immune responses that preclude long-term transgene expression (102)(103)(104)(105)(106)(107), investigation of AAV vectors as a possible alternative is highly desired.

1.2 Pulmonary fibrosis

1.2.1 Epidemiology, clinical aspects and pathobiology

Fibrotic diseases such as liver cirrhosis, systemic sclerosis, chronic kidney disease and cardiac or pulmonary fibrosis share the common characteristic of a dysregulated or persistent wound healing process that often occurs due to repetitive injury and tissue damage of various causes. Idiopathic pulmonary fibrosis (IPF) is a chronic progressive disease, where the excessive production of extracellular matrix (ECM) within the pulmonary interstitium leads to the loss of alveolar functionality, often resulting in respiratory failure and ultimately death. Changes in IPF diagnostic criteria led to strongly fluctuating estimates on the epidemiology; however, according to data from population-based medical recording in Minnesota and health insurance database analysis, the prevalence of IPF ranges from 14.0 to 63.0 cases per 100,000 and the incidence lies between 6.8 and 17.4 new annual cases per 100,000 (108). IPF occurs mainly in middle-aged to older patients (average onset at age 66) and is often only diagnosed at advanced disease stage, due to the rather unspecific symptoms of chronic unproductive cough and shortness of breath. The mean survival following diagnosis is two to five years. Primary diagnosis is based on high-resolution computed tomography of the chest to examine the lung for parenchymal destruction.
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(“honeycombing”) and scarring. Histological examination of lung tissue biopsies for signs of usual interstitial pneumonia (UIP), i.e. heterogeneous patches of healthy, actively scarring and fibrotic tissue along with the accumulation of collagen-producing fibroblasts surrounded by hyperplastic alveolar epithelial type II cells (so-called fibrotic foci), can additionally help in clinical decision making (109). Regarding prognosis, forced vital capacity (FVC) measurement (i.e. the volume of air, a person can exhale upon a maximum inspiration) is considered most reliable (110). Moreover, the annual decline in FVC was also used as the primary endpoint in recent clinical trials for Pirfenidone and Nintedanib (111)(112), the most efficacious therapeutic agents available for the treatment of IPF to date. However, despite these successes, therapeutic treatment can only decelerate disease progression and no options are available for curing IPF. Even the ultima ratio of treatment, i.e. lung transplantation, only results in a five-year survival rate of 44 % (113).

Despite the fact that IPF develops for unknown reasons, it is generally accepted that repeated injury to the alveolar epithelium is a major factor that triggers fibrosis. Persistent irritants include allergens, viral infections, environmental toxins but also radiotherapy and chemotherapeutic agents such as Bleomycin (114)(115)(116)(117). Moreover, a small proportion of about three percent of all IPF cases are familial and associated with polymorphisms in either of the genes encoding Tumor necrosis factor α (TNFα), Transforming growth factor β1 (TGFβ1), Surfactant protein C (SFTPC) (118)(119)(120) or the promoter of Mucin 5B (MUC5B) (121).

Following epithelial or endothelial injury by one of the irritants described above, these cells release inflammatory cytokines and chemokines, which induce an anti-fibrinolytic clotting cascade, to prevent blood loss and to regenerate barrier function (122). The degranulation of aggregated platelets that plug the wound induces blood vessel dilation and increases permeability for the invasion of inflammatory leukocytes, most prominently neutrophils accompanied by macrophages and lymphocytes. Increased tissue permeability is achieved by matrix metalloproteinases (MMPs), for instance MMP-2 and MMP-9, which cleave extracellular matrix components such as collagens (123). Inflammatory mediators released by the recruited immune cells perpetuate and amplify inflammation and further stimulate fibroblast proliferation and their differentiation to myofibroblasts, which are contractile cells that express high levels of α-smooth muscle actin and generate and deposit ECM-components, including fibronectin and type I collagen (124). During normal wound healing, myofibroblasts facilitate wound healing by pulling the wound together and by producing a provisional matrix that serves as a scaffold for the migration of epithelial and endothelial cells that regenerate the damaged tissue – a process known as wound contraction and -closure. Afterwards, the matrix is mobilized by fibrinolysis and repair- and immune cells die due to apoptosis. However, in IPF, regulation of normal wound healing, including myofibroblast
apoptosis seems to be disturbed (125) and myofibroblasts continue producing scar tissue, thereby representing the major effector cell type involved in tissue remodeling. A scheme of key events during normal and pathological wound healing is shown in Figure 3.

**Figure 3: Normal and pathological wound healing**

Wound healing can be roughly divided in four stages: (1) an initial phase of clotting and coagulation, where inflammatory mediators secreted by injured epithelial cells induce a clotting cascade, resulting in platelet activation and clot formation. (2) a phase of inflammation, where leukocytes attracted by inflammatory chemokines and cytokines secrete pro-fibrotic mediators that trigger (3) fibroblast migration, proliferation and differentiation to myofibroblasts, which might originate from circulating bone marrow (BM)-derived fibrocytes, epithelial cells that undergo epithelial-to-mesenchymal transition (EMT), resident fibroblasts or pericytes (not shown). (4) Extracellular matrix (ECM) components produced and deposited by myofibroblasts build a provisional matrix that either serves normal wound contraction or results in fibrosis. Fibrosis might occur if any of the processes depicted in this scheme are dysregulated or when the tissue-damaging insult persists. Figure reprinted from Wynn TA, JEM 2011 (126).

Myofibroblasts can potentially originate from different progenitor cells. Candidate precursor cells include epithelial cells, endothelial cells, circulating fibrocytes, pericytes and resident fibroblasts. Epithelial cells, which de-differentiate to acquire a mesenchymal phenotype in a process called epithelial-to-mesenchymal transition (EMT) could be an important source for myofibroblasts in fibrotic diseases. EMT is a well-studied process that occurs during embryogenesis, wound healing and tumor metastasis, and is characterized by a loss of polarity (i.e. apical and basal orientation of the cell), loss of attachment and cell-cell interactions and therefore acquisition of a migratory phenotype (127). On a molecular level, EMT is characterized by a downregulation of epithelial markers and cell-adhesion molecules such as E-cadherin and upregulation of mesenchymal markers such as N-cadherin and vimentin (127). Similarly, endothelial cells were suggested to undergo transition to a mesenchymal phenotype, what was called EndoMT (128). Moreover, also fibrocytes, i.e. circulating mesenchymal cells from the bone marrow were discussed as a source for myofibroblasts (124). However, despite a strong focus on the aforementioned cells during the last years, especially epithelial cells, recent research questions their importance regarding the accumulation of myofibroblasts (129)(130) and rather suggest indirect involvement in fibrosis.
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based on cell-to-cell signaling activities rather than progenitor function. In contrast, pericytes and resident fibroblasts have lately gained attention and are strongly indicated as the cellular precursors for myofibroblasts. Pericytes are mesenchymal cells that, together with endothelial cells are located at a common basement membrane, lining microvascular structures such as capillaries (124)(130). Studies in various organs suggest that upon injury, pericytes mobilize and proliferate to become myofibroblasts, and fate tracing studies in the lung demonstrated similar results for pulmonary fibrosis, resulting in about 45% pericyte-derived myofibroblasts (130). The remaining 55% might originate from various sources, including resident fibroblast, which are ECM component-producing cells located within the connective tissue in close proximity to epithelial and endothelial cells (124).

Similar to the controversy regarding potential myofibroblast precursors, the role of inflammation during the development and progression of pulmonary fibrosis is highly controversial (131)(132). While there is consensus about inflammation being a normal result of tissue damage and epithelial injury during a wound healing response, its role during disease maintenance and progression is unclear. On the one hand, analyses of patient samples showed heterogeneous lung areas of both, chronic inflammation and fibrosis, suggesting the involvement or even prerequisite of inflammation for fibrosis to develop (132). On the other hand, histologically identified inflammation is moderate, there is no correlation between inflammation and disease stage or outcome, and anti-inflammatory pharmacological treatment is largely inefficacious (133). Therefore, one hypothesis is that pro-fibrotic mediators that are sequestered in fibrotic microenvironments (i.e. fibroblast foci) might inhibit apoptosis of immune cells normally passing through the tissue, thereby facilitating their accumulation, which might be falsely interpreted as inflammation (131). Moreover, there is the theory that inflammation during late-stage fibrosis might even be beneficial, because inflammatory cells could assist in controlling the increased cellular proliferation and phagocytose cellular debris (134).

The nature of a wound healing response involves many different structural and immune cell types following an initial insult, coagulation, wound healing and -closure, which only adds to the complexity of inflammatory cytokines, chemokines and growth factors involved in fibrosis pathobiology (134)(126): During injury, damaged epithelial and endothelial cells release inflammatory factors triggering vasodilation and matrix mobilization to facilitate platelet and leukocyte invasion. Moreover, release of damage-associated molecular patterns (DAMPs) such as nuclear and cytoplasmic proteins and DNA can perpetuate inflammation, for instance by triggering TNFα and IL-6 release and further cause autoimmune reactions. Different environmental triggers such as chemical pollutants and pathogens (see above) can additionally
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alter the inflammatory environment. Depending on the cellular composition of the immune cell infiltrate, cytokine profiles might show a more pronounced pro-inflammatory type I (e.g. TNFα, interferon γ, IgG2 antibody) or wound healing-associated type II (e.g. IL4, IL5, IL13) signature. Growth factors like connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and TGFβ1 regulate angiogenesis to compensate for chronic injury-induced damage to the vasculature. Further factors influencing the complex fibrotic process include oxidative stress (e.g. by neutrophil-mediated release of reactive oxygen species) and the increasingly stiffened ECM, which is sensed by integrins and other ECM-cell-attachment proteins, thereby regulating cellular behavior in a process termed mechanotransduction (135), which is elaborated on in more detail in the next paragraph.

1.2.2 Transforming growth factor β1 (TGFβ1) and its role in pulmonary fibrosis

1.2.2.1 TGFβ1 synthesis, activation and role in development and disease

Among many different cytokines and growth factors implicated in the development and progression of fibrosis, TGFβ1 gained particular attention, due to its ability to stimulate the production of collagen and other ECM-components (136)(137), to induce EMT (138)[139], fibroblast proliferation and transition to myofibroblasts (140), and further processes linked to inflammatory cell recruitment, immune regulation and wound healing (141)(134). Moreover, TGFβ1 is upregulated in human IPF tissue samples (142)[143] and adenoviral overexpression of TGFβ1 in animal models is sufficient to cause pulmonary fibrosis (102)(144). Finally, mice with a knockout of SMAD3, a critical downstream mediator of TGFβ signaling (see 1.2.2.2), were protected from both TGFβ1- and Bleomycin-induced fibrosis (145)[146].

TGFβ1 is one of three TGFβ isotypes and is expressed by most cells of the body. TGFβ1 plays a crucial role in a great variety of processes, including organ development, regulation of cell growth, proliferation and differentiation, immune regulation, angiogenesis and wound healing (147)[141][148]. Each of the isoforms is expressed from a unique promoter and shows differential tissue localization (149)[143]. All three isoforms are required for development, as the genetic deletion of each of the respective genes in mice was lethal: While TGFβ1-mutant mice died approximately three weeks after birth, due to multi-organ inflammation and autoimmune reactions, resulting in organ failure, wasting and therefore death (150), prenatal or perinatal death was observed for TGFβ2-deleted mice (151). In that case, developmental defects in various organs and tissue structures such as lung deformation were reported. TGFβ3 deletion in mice resulted in cleft palate and lung deformation, which led to death few hours after birth (152). Interestingly, given that each of the mutant mice had a distinctly different phenotype, the results
indicate that each of the TGFβ isoforms play numerous important, non-compensable roles during development.

TGFβ1 is produced, secreted and activated following a relatively well-defined process (153)(154) (Figure 4): TGFβ1 is synthesized as an inactive precursor protein (pre-pro-TGFβ1) that contains a N-terminal signal peptide upstream of an additional peptide called latency-associated peptide (LAP). After removal of the signal peptide in the endoplasmatic reticulum, two latent pro-TGFβ1 molecules build a homodimer by disulphide bridging and LAP gets cleaved off by furin convertase in the secretory vesicle. However, the LAP protein remains non-covalently attached to the mature TGFβ1 homodimer, enclosing it in a clamp- or straitjacket-like fashion, thereby preventing its interaction with TGFβ receptors. The LAP-TGFβ1 complex is termed small latent complex (SLC). Most cells secrete the SLC bound to the latent TGFβ-binding protein LTBP, thereby forming the large latent complex (LLC). There are four currently known LTBP5s, each of which harbors N-terminal binding epitopes for various ECM components, including fibronectin. Covalent ECM-binding is then mediated by transglutaminase. The C-terminal domain binds to fibrillin-1, therefore connecting the LLC with elastic microfibrils. By binding of these structural components, latent TGFβ1 is stored in the extracellular niche; moreover, in addition to storage, LTBP- and transglutaminase-antagonizing experiments showed that LTBP-mediated ECM binding is necessary for efficient TGFβ1 activation (155)(156).

For activation, the LLC needs to first be liberated from the ECM, which was shown to be mediated by inflammatory proteases such as elastase that cleave fibrillin-1, and by enzymes that cleave LTBP, for instance BMP-1 (153). Release of mature TGFβ1 requires liberation from the LAP, which can be either mediated by LAP-proteolysis by MMP2, MMP9 (157), membrane-type MMPs (158), plasmin and thrombin (159) or tryptase (160), by binding of thrombospondin-1 to the LAP, thereby inducing a conformational change (161) or by mechanical forces mediated by integrins, which can bind a RGD motif within the LAP (162)(163)(164)(165). In the latter case, LTBP-binding of the ECM provides resistance against pulling forces mediated by integrins of contractile cells. Recent research showed that increasing stiffness of the ECM – as it is the case in (pulmonary) fibrosis – promotes TGFβ1 activation by lowering the force required for the integrin- and cellular contraction-mediated pulling and subsequent “opening” of the LAP-clamp to release mature TGFβ1 (166). These results established a link between the previous findings showing that fibroblasts trans-differentiate to myofibroblasts in a rigid environment (i.e. tight collagen vs. loose collagen) (167)(168), which was shown to be mediated by TGFβ1 and dependent on integrin signaling via focal adhesion kinase (169). Moreover, this mode of mechanical activation also provides a potential explanation for the continuous presence of TGFβ1 expression during IPF in
human patients. Specifically, increasing matrix stiffness during the progressive disease course could perpetuate pro-fibrotic processes by continuous mechano-activation of TGFβ1. Therefore, the finding that cells sense mechanic signals of their environment via interaction with the ECM and respond by signaling events, including TGFβ1 activation, supports the novel concept of mechanotransduction (170)(135).

**Figure 4: Synthesis and activation of TGFβ1**

(a) 1) TGFβ1 is synthesized as a pre-pro-protein, whose N-terminal signal is cleaved off, yielding 2) pro-TGFβ1. Two pro-TGFβ1 monomers assemble a homo-dimer. Furin convertase cleaves the dimer and 3) the disulphide bond-stabilized latency-associated protein (LAP) wraps around mature TGFβ1 in a non-covalent way, forming the small latent complex (SLC), 4) which is usually secreted covalently bound to the latent TGFβ1 binding protein (LTBP), forming the large latent complex (LLC). The LTBP can bind microfibrils (fibrillin-1) and ECM components, including fibronectin via specific binding sites within its C- and N-terminus, respectively. ECM-links can be covalent by cross-linking activities of transglutaminase. (b) 5) Elastase-mediated cleavage of fibrillin-1 can mediate LLC detachment and 6) the ECM-bound hinge region of the LTBP can be cleaved by BMP-1. 7) To release mature TGFβ1, the LAP is either proteolytically cleaved, which can be mediated by MMP2, MMP9, membrane-type MMPs, plasmin, thrombin and tryptase, by binding of thrombospondin-1 to the LAP or by integrin-mediated pulling forces, which bind to RGF motifs (blue regions in 6)) within LAP and open the LAP “clamp” to release TGFβ1 (see text for details). 8) Active TGFβ1 can then bind to its receptors TGFBRII and ALK5. Figure reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, ten Dijke P and Arthur HM, Extracellular control of TGFβ signalling in vascular development and disease. (153), copyright 2007.
1.2.2.2 TGFβ1 signaling

The key steps of canonical TGFβ signaling are well-documented (148). There are three different TGFβ receptors, type I, type II and type III. Type III, which is meanwhile known as betaglycan, is not directly involved in canonical TGFβ signaling but might serve as a ligand reservoir for the type I and II receptors (171). As the TGFβ superfamily includes Bone morphogenetic proteins (BMPs), Growth and differentiation factors (GDFs), Anti-müllerian hormone (AMH), Nodal, Activin and the different TGFβ isoforms, different TGFβ type II receptors, which are the receptors initially bound, are available for each ligand. For the sake of simplicity, the following descriptions will largely focus on TGFβ1 as a ligand. After binding of TGFβ1 to a type II receptor dimer, a type I receptor dimer, which is also known as ALK5, is recruited and phosphorylated by the serine/threonine kinase activity of the type II receptor. After internalization of the receptor-ligand complex in clathrin-coated pits, the endosomally present SMAD anchor for receptor activation (SARA) binds the type I receptor. By orienting their spatial position, it facilitates the binding and activation of SMAD2 and SMAD3, which belong to the family of receptor-regulated SMADs (R-SMADs, SMAD 1, 2, 3, 5 and 9), to the type I receptor. Moreover, SARA also recruits different endocytosis-mediating factors relevant for receptor internalization (172). Within the endosome, the type I receptor phosphorylates SMAD2 and 3, which induces a conformational change that triggers their dissociation from SARA and the receptor. Subsequently, SMAD2 and 3 bind to SMAD4, a common SMAD (co-SMAD) and adapter protein, thereby forming heterodimers, -trimers or -hexamers, which subsequently translocate to the nucleus. In the nucleus, the SMAD-complexes can bind to several additional factors and act as transcription factors to modulate gene expression of genes harboring a SMAD-binding DNA element (SBE) by direct binding to their DNA. The various binding partners along with the cellular context and TGFβ and SMAD concentrations explain the diverse, pleiotropic and sometimes contrary nature of TGFβ-mediated effects.

The canonical signaling pathway can be modulated by various ways involving several mediators and molecular targets. For instance, SMAD recruitment and signaling can be promoted by SUMOylation, by Endofin (a potential modulator of endosomal trafficking), Axin (a Wnt signaling modulator and tumor suppressor) and Dab2 (that directly interacts with SMADs) (173)(174). In contrast, SMAD-mediated signaling can be decreased by proteasomal degradation upon SMAD2 ubiquitination by the ubiquitin ligases SMURF2 and NEDD4, the latter of which also ubiquitinates the type I TGFβ receptor (175)(176). These effects can be antagonized by Ubiquitin carboxyl-terminal hydrolase 15 (USP15), which promotes deubiquitination of SMAD2, SMAD3 and/or the type I TGFβ receptor (177)(178). Inhibitory SMADs (I-SMADs) such as SMAD7, whose expression is induced by R-SMADs, can regulate TGFβ signaling via negative feedback by various means: SMAD7 can actively recruit SMURF1 and -2 to ubiquitinate the type I receptor for degradation (179)(180).
Moreover, it competes with R-SMADs for type I receptor interaction, thereby attenuating their phosphorylation (148). Finally, SMAD7 induces Salt inducible kinase (SIK) to promote TGFβ type I receptor internalization and degradation (181). In addition to SIK, the endosomal sorting nexin 25 (SNX25) was shown to facilitate the lysosomal degradation of the type I receptor (182). In turn, RAB11 can contribute to the recycling of internalized type I receptor to be re-exposed on the cell membrane and RAP2 was shown to increase signaling by competing with the SMAD7-SMURF1 complex (183).

In addition to canonical SMAD-mediated signaling, TGFβ1 utilizes several other non-SMAD interaction partners to modulate cellular responses, including MAP kinase pathways via ERK and JNK, Rho-like GTPase cascades and PI3K/AKT signaling (184). TGFβ1 can induce tyrosine phosphorylation of the type I and II receptors, which can further trigger the phosphorylation of SHC. By recruiting GRB2 and SOS, the SHC/GRB2/SOS complex can activate ERK signaling via RAS, RAF (MAP3K) and MEK (MAPKK). ERK is not only required for TGFβ1-induced EMT by promoting adherens junction disassembly, but can also regulate SMAD activity by direct phosphorylation of SMAD1-3. Another crucial pathway for EMT is mediated by TRAF6 interaction with the TGFβ receptors, which induces TRAF6-polyubiquitinylination. By recruiting TAK1, TRAF6 can then activate JNK/p38 signaling, which, in conjunction with SMAD signaling, regulates TGFβ-mediated apoptosis induction. An additional pathway requires the GTPase RhoA, which can be activated by TGFβ1 to induce stress fiber formation. On the other hand, via TGFβ receptor type II-mediated PAR6/SMURF1 recruitment, RhoA can be ubiquitinated and degraded, thereby enabling tight junction dissolution and subsequent EMT. Moreover, interaction with the Rho-like GTPase Cdc42 can additionally regulate cell-adhesion by direct interaction with the tight junction protein occludin. Finally, possibly through a direct interaction between TGFβ receptors and PI3 kinase, AKT can be phosphorylated and induce mTOR-mediated S6 kinase activation, thereby influencing protein synthesis, EMT and fibroblast proliferation. In contrast, AKT can also antagonize TGFβ effects by inhibiting SMAD3 phosphorylation and preventing the nuclear localization of FoxO, which mediates TGFβ1’s growth inhibiting effects by suppressing c-myc.

Besides the interaction partners described here, TGFβ likely uses various additional routes to control its complex downstream functions, which is also suggested by TGFβ receptor interaction mapping that identified more than 100 receptor complex-associated proteins (185). Moreover, there is an overwhelmingly complex cross-talk with other prominent pathways including Wnt, Hedgehog, Notch, Interleukin, Interferon, TNF and NFκb signaling, based on feedback loops, direct and indirect interactions and synergistic as well as antagonistic effects of TGFβ itself or downstream molecules as the ones described above (186). In summary, the plethora of effects
modulated by TGFβ signaling is strongly context-dependent, which includes the developmental stage, cell types, physiological status and available co-factors.

1.2.3 Animal models of pulmonary fibrosis

Efforts to replace, reduce and refine animal studies (which is known as the “3R” principle) have been ambitiously pursued and led to remarkable innovations such as the “lung on a chip” (187). Moreover, by using cellular models to mechanistically mimic disease processes such as epithelial-to-mesenchymal transition and fibroblast-to-myofibroblast transition in vitro, the number of animal studies can additionally be reduced. Nevertheless, to investigate the interplay of pathways, signaling events and cross-talk across several neighboring cell types in a complex, three-dimensional tissue environment as well as for pharmacological profiling of drug candidates, so far animal models are hard to substitute for. A range of different animal models are available for pulmonary fibrosis and thorough characterization of each of these models is a prerequisite to ensure their reasonable use for a given scientific issue. In essence, there are four different approaches, relying on lung injury, aging, “humanization” by transfer of IPF cells or transgenic cytokine expression, each of which will be briefly introduced in the following (188)(189).

The first model relies on intratracheal asbestos instillation, which results in fibrosis on day 7 that develops until day 14 and usually persists or even progresses with time (188)(189). Asbestos fibers injure the alveolar epithelial cells (AEC), cause oxidative stress and are evident from asbestos bodies in the lung tissue. Cellular infiltrates are characterized by macrophages, lymphocytes, eosinophils and neutrophils. Unlike many other models, in the asbestosis model fibroblast foci develop, albeit at lower levels than in human IPF.

An alternative model utilizes intratracheal silica installation, thereby mimicking silicosis, where fibrosis develops around deposited silica particles in the tissue, 14-28 days after administration (188)(190). Fibrosis is persistent; however, the degree can vary strongly depending on the silica material used. A special feature of this model is the strong impact of the NALP3 inflammasome along with a persistent toxic, inflammatory response. A finding for both asbestos- and silica-induced fibrosis is that longer exposure, preferentially by inhalation instead of intratracheal instillation, induces a more slowly evolving disease, which, however, might more closely mimic the human phenotype, including disease manifestation in more peripheral areas of the lung.

Intratracheal fluorescein isothiocyanate (FITC) administration represents a third lung injury-based model, characterized by alveolar and vascular permeability/leakage as well as edema and inflammation, which turns into fibrosis after 14-21 days, which can persist for months (188)(189). Specialties of this model include a strong type 2 T-helper cell (TH2) component and the possibility
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to study the spatial link between injury and fibrosis development, as FITC can be easily imaged. Depending on the source and size of FITC particles, which is determined by the degree of sonication used, toxicity and fibrosis development can vary.

The most widely established model is intratracheal Bleomycin administration, which is based on the finding that some Bleomycin-treated patients develop fibrosis in the clinic (117). Bleomycin is a small peptide harboring a DNA-binding and Fe^{2+}-binding region. Oxidation of Fe^{2+} to Fe^{3+} results in the simultaneous reduction of oxygen to free radicals, which induce DNA strand breaks and RNA degradation, resulting in oxidative stress and alveolar epithelial cell death immediately after instillation (191)(188)(192). The subsequent strong inflammatory phase evolves into fibrosis, which, however, often resolves after several weeks, although reports are controversial. Therefore, repetitive administration of lower doses has been established to mimic the repetitive injury probably underlying IPF pathogenesis, which results in AEC hyperplasia and persistent fibrosis. However, repeated dosing is laborious, costly and might induce larynx injury, which is why the single-application protocol remains most widely used. Alternative routes of administration include the intraperitoneal and intravenous route, which result in initial vascular endothelial cell damage.

Similar to Bleomycin, radiation-induced fibrosis is a clinically observed situation, based on injury of the respiratory tract, likely involving free radical-induced DNA damage (188)(193). Following radiation limited to the thorax, inflammation evolves into fibrosis evident by approximately 5 months. A specific feature of this model might be the vascular remodeling observed, which is similar to clinically observed pulmonary hypertension.

A different approach to induce “targeted” injury was achieved by generating transgenic mice that expressed the diphtheria toxin receptor (DTR) under the control of the AEC type II cell-specific surfactant protein C-promoter and repeated intraperitoneal administration of diphtheria toxin (188)(194). Despite AEC hyperplasia and subsequent fibrosis that persisted through day 28, a disadvantage of this model lies in the rather high mortality and its use is probably restricted to investigating the cellular responses that turn epithelial injury into fibrosis.

Aging is also implicated in pulmonary fibrosis, given that the average age of disease onset is 66. Mice at increasing age showed higher susceptibility to Bleomycin-induced fibrosis as did mice prone to cellular senescence (188)(195). Moreover, when aged mice were infected with γ-herpesvirus-68, they developed fibrosis, which was in contrast to young mice (196). Notably, herpesviral genomes are often found in IPF patients, suggesting that viral infections could facilitate fibrosis development in aged people. Finally, male aged mice seem to be more prone than female animals, recapitulating the male predominance of IPF. Despite the fact that the use of
aged mice (older than 12 months) could improve fibrosis disease models, their use is associated with high costs and enormous efforts, preventing broader application.

Another approach is the “humanization” of mice by intravenous administration of human IPF-derived fibroblasts into NOD/SCID mice that are largely immunocompromised with regard to both innate and adaptive immunity (188)(197). Unlike fibroblasts from healthy lungs, IPF fibroblasts caused persistent, lung-specific alveolar remodeling evident from approximately 5 weeks after administration, thereby proving their pathogenicity. In spite of the model’s usefulness for studying differences between fibroblasts of different origin and potentially the evaluation of patient-targeted therapy, the model is very artificial with regard to largely absent immune functions and moreover limited by the high costs of respective mice.

Finally, cytokine overexpression has proven useful to study the impact of certain proteins and pathways in fibrotic disease biology. IL1β, IL13, TNFα and TGFβ1 are among the genes that were overexpressed (198)(199)(200)(102), using either Adenovirus vectors (AdV) or inducible systems (188). Intratracheal administration of AdV-IL1β induced pulmonary fibrosis associated with progressive collagen deposition (day 21-60), early inflammation characterized by neutrophils and macrophages and elevated levels of TGFβ1 and platelet-derived growth factor (PDGF). AdV-TNFα vectors also induced fibrosis with a similar inflammatory profile and upregulation of TGFβ1. However, a second study demonstrated TNFα-mediated attenuation of both Bleomycin- and TGFβ-induced fibrosis, therefore suggesting a complex, probably context- and timing-dependent function of TNFα. Transgenic IL13 overexpression using the club cell-specific CC10 promoter also induced fibrosis, which, however, was shown to be dependent on TGFβ1. Lastly, AdV-mediated or doxycycline-inducible, CC10 promoter-driven overexpression of TGFβ1 also induced fibrosis, characterized by massive collagen-production and alveolar epithelial death. Both approaches led to persistent scarring, albeit at different kinetics. While AdV-mediated effects were rapid, reaching a doubling of collagen at day 14, a similar effect was reached in the doxycycline-induced model after 2 months of TGFβ1 induction, likely due to lower levels of TGFβ1 expression. Further points to note are that AdV-mediated TGFβ1 expression was associated with early inflammation and that transgene expression peaked seven days after virus administration but was largely absent on day 21, most probably due to T-cell-mediated clearance of vector-transduced cells.

In summary, none of the models fully recapitulates all IPF-relevant features. Nevertheless, especially the more persistent and ideally progressive models should allow studying critical disease processes such as fibrogenesis. In addition to injury-based models, rapid-to-setup transgenic models, allowing examining long-term gene function, are also desired to dissect the molecular pathways critical in disease maintenance and progression.
1.3 Aim of the thesis

Adeno-associated virus vectors are powerful tools for the modulation of gene expression in preclinical research and therapeutic applications by targeted delivery of cDNA, shRNA or miRNAs to cells and tissues. However, their use for functional studies in the context of lung disease research remains sparse and AAV-based pulmonary disease modeling has not been proven to date. In this thesis, it should therefore be evaluated, whether AAV vectors, specifically AAV6.2 – an AAV variant reported to demonstrate particularly high lung transduction efficiency – are suitable to setup pulmonary disease-related models in mice.

For this purpose, the efficiency, cellular tropism and stability of AAV6.2-mediated lung gene transfer is studied. Moreover, immunogenic effects of AAV vector administration to the lung will be compared with those of a second-generation Adenovirus-5 vector, the most commonly used tool for vector-based studies in the lung so far. AAV6.2’s suitability for disease modeling will be investigated by overexpression of TGFβ1 in the lung of mice and the subsequent in-depth characterization of phenotypic and transcriptional changes in comparison with the widely established model of Bleomycin-induced pulmonary fibrosis. Resulting mRNA and miRNA profiles are examined for common disease signatures to identify novel candidate genes and miRNAs that might play important roles in disease onset and maintenance. Finally, the possibility to test small molecule-drug candidates in the AAV-TGFβ1 model will be examined.

In the last chapter of the thesis, it will be investigated whether artificial, self-cleaving riboswitches can be used to control transgene expression during AAV vector production and after AAV-mediated transgene delivery to target cells. Such riboswitch-AAV vectors could present an innovative approach for various technological and functional applications, including AAV gene therapy.
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2 Results

Modeling pulmonary fibrosis by AAV-mediated TGFβ1 expression – a proof of concept study for AAV-based disease modeling and riboswitch-controlled vector production
2.1 Evaluation of AAV6.2 as a tool for lung gene transfer

2.1.1 Analysis of the transduction profile of AAV6.2

Adeno-associated virus (AAV) vectors have been successfully used to transduce primary human airway epithelial cells in vitro (81)(92) and to express reporter and functional genes in the lung of mice (see introduction). In a comparative study using several AAV serotypes and capsid variants (81), AAV6.2, an AAV6-based capsid variant carrying a F129L mutation in the VP1 capsid protein, showed the highest transduction rate as compared to other AAV serotypes, such as AAV5 and AAV9. Using β-galactosidase and green fluorescent protein (GFP) reporter gene-carrying AAV5, -6, -6.2 and -9 vectors, we confirmed and extended these results. Specifically, we found that AAV6.2 vectors demonstrated higher transduction efficiency than AAV5 and AAV6 on primary bronchial epithelial cells, small airway epithelial cells, bronchial smooth muscle cells and lung fibroblasts. Moreover, after intratracheal (i.t.) administration to the lung of mice, AAV6.2 showed superior transduction efficiency in comparison to AAV5 and AAV9 (Bachelor thesis work, data not shown).

In order to study the distribution and stability of AAV6.2-mediated transgene expression in the lung of mice, a protocol for anti-GFP immunohistochemical (IHC) staining using formalin-fixed, paraffin-embedded (FFPE) lung tissue was established. Using this protocol, FFPE lung sections of mice that had received AAV6.2-CMV-GFP vector via i.t. application were examined for GFP expression at different time points after vector administration. From two out of five treated animals cryo-lung sections were prepared instead of FFPE sections and GFP fluorescence was assessed directly by fluorescence microscopy. Both, direct fluorescence and anti-GFP immunostaining revealed a distinct pattern of GFP expression in the lung of mice. Specifically, GFP expression was located in bronchial and small airway epithelial cells along with an additional, yet unidentified cell type in the alveolar tissue (Figure 5).

To identify the alveolar cell type transduced by AAV6.2, the GFP-positive cells were analyzed in more detail by microscopy. Microscopic examination revealed that the GFP-positive cells often had a cuboidal shape and were predominantly located at alveolar branching points, thereby suggesting that these cells might be type II alveolar epithelial cells (AECs). Contrary, type I AECs have a rather thin and flat morphology and line the alveolus (Figure 6a). To confirm this assumption, an IHC staining protocol for Surfactant associated protein C (SFTPC), a unique marker of type II pneumocytes, was established. Alternately anti-GFP- and anti-SFTPC-stained serial lung tissue sections of AAV6.2-CMV-GFP treated animals showed that positively stained cells had similar morphology and tissue location in both sections, thereby confirming that AAV6.2 vectors transduce type II alveolar epithelial cells (Figure 6b).
Figure 5: Analysis of the pulmonary transduction pattern of AAV6.2

(a) IHC analysis of GFP expression in FFPE lung sections of mice 28 days after i.t. application of PBS or $1 \times 10^{11}$ vg AAV6.2-CMV-GFP. Shown is the whole left lung lobe. (b) GFP-positive structures, which were defined as those exceeding a specified color intensity threshold identified by computed image analysis of the GFP-IHC staining shown in (a), were automatically colored in red for better visualization. 3.5-fold enlarged details are shown in the separate boxes. Direct fluorescence micrographs of cryo-lung sections are additionally shown in (b). Representative images of n= 5 animals per group are shown.
2.1.2 Immunological profile and long-term transgene expression

Due to their natural pulmonary tropism, Adenovirus-5 (Ad5) vectors until now represent the most frequently used vectors for functional studies in the lung (101), including modeling of pulmonary fibrosis by overexpression of TGFβ1 (102). However, their application is associated with several limitations, among them the need for biosafety level 2 environment and, more importantly, immunological responses triggered by Adenovirus vectors in vivo, which compromises stable transgene expression and can potentially alter relevant readouts in preclinical studies. In fact, it has been shown that inflammation mediated by Ad5 control vectors predisposes mice to fibrosis (102). In contrast, AAV vectors are regarded as non-pathogenic and have been shown to only induce moderate immune responses in vivo, thereby enabling prolonged transgene expression after a single vector application. While respective findings were reported for several different AAV serotypes and target tissues, until now, no systematic analysis of immune responses and long term transgene expression has been reported in the context of pulmonary gene transfer.

Therefore, to analyze AAV6.2's immunogenicity in the lung of mice, AAV6.2 control vectors were produced that only contained non-coding “stuffer”-DNA (hence referred to as AAV6.2-stuffer) and Ad5-stuffer vectors were obtained commercially. Following intratracheal administration of these vectors, the early immune response was characterized over a period of 1 week.
Results

After i.t. delivery of either $1 \times 10^{11}$ vg AAV6.2-stuffer or $1 \times 10^8$ infectious units (IU) Ad5-stuffer vectors, immune responses were assessed over a period of one week. (a) Total immune cell infiltration and differential analysis in BAL samples. (b) qPCR analysis of host defense-associated genes. (c) Detection of inflammatory BAL cytokines using ELISA. n=3 (PBS) and n=5 (Ad5/AAV6.2) animals per group. Mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001, #p<0.05, ##p<0.01, ###p<0.001. Asterisks indicate statistical significance relative to both AAV6.2 and PBS in (a) and (c). In (b) asterisks refer to AAV6.2 and hash marks refer to PBS.

The results show that the total immune cell counts measured in the bronchoalveolar lavage (BAL) of mice increased sharply at 12 h after application of Ad5, which was mainly due to neutrophils (Figure 7a). In contrast, there was no significant increase with AAV6.2 or the vehicle control at any time point. In addition, Ad5 application induced an increase in the Major histocompatibility
complex I (MHC-I) genes H2D1/K1/L, as well as the Toll-like receptor signaling genes TLR3 and MYD88 in total lung RNA samples shortly after viral vector application, thereby indicating activated host defense (Figure 7b). Moreover, TLR9 expression increased 1 week after Ad5 application, accompanied by a reoccurring increase of MYD88 (Figure 7b). Notably, BAL analysis revealed an Ad5-specific increase of the inflammatory cytokines IL1β, IL6 and TNFα immediately (6-12 h) after vector application (Figure 7c). Consistent with the influx of neutrophils, the levels of the neutrophil chemoattractant KC (CXCL1) were also strongly increased at these early time points (Figure 7c). In stark contrast, AAV6.2 did not induce any of these changes and behaved as immunosilent as the PBS control.

Figure 8: T-cell response following i.t. administration of AAV6.2- or Ad5-stuffer vectors

After i.t. delivery of either $1 \times 10^{11}$ vg AAV6.2-stuffer or $1 \times 10^{8}$ IU Ad5-stuffer vector particles, immune responses were assessed over a period of one week. (a) Flow cytometry analysis of CD4$^+$ and CD8$^+$ BAL lymphocytes at 168 h after virus application and (b) quantitative presentation thereof, where each bar represents the pooled BAL of n=3 (PBS) and n=5 (Ad5/AAV6.2) animals, respectively. (c) Detection of BAL interferon gamma levels using ELISA. n=3 (PBS) and n=5 (Ad5/AAV6.2) animals per group. Mean +/- SEM. ***p<0.001.

Antiviral responses are usually biphasic, where an immediate inflammatory reaction is followed by a cytotoxic T-cell response clearing virus-infected cells. Notably, neutrophil and lymphocyte cell counts increased again at 168 h after Ad5 treatment, thereby suggesting the beginning of this second phase (Figure 7a). Flow cytometry analysis of pooled BAL samples revealed that indeed, both CD4+ and CD8+ T-lymphocytes were increased at 168 h after Ad5 treatment but not in
Results

AAV6.2 treated animals (Figure 8a,b). Moreover, strongly elevated protein levels of the T-cell cytokine IFN-γ in the BAL of Ad5-treated animals further substantiated these findings (Figure 8c). Taken together, these results demonstrate that, contrary to a commonly used Ad5 vector, AAV6.2 did not induce any detectable inflammatory responses after application to the lung of mice.

Following these results, it was finally assessed whether the favorable immunogenic profile of AAV6.2 would allow for long-term transgene expression. To this end, AAV6.2-CMV-GFP vectors were instilled to the lung of mice and GFP expression was examined by anti-GFP IHC staining 4, 8 and 16 weeks after a single vector administration. The IHC results show that GFP was expressed robustly and in accordance with the previously observed transduction pattern (Figure 5) for the full tested time period of 4 months (Figure 9).

Figure 9: AAV6.2-mediated long-term transgene expression in the lung of mice
Immunohistochemical analysis of GFP expression in FFPE lung sections of mice, 4, 8 and 16 weeks (wks) after i.t. administration of either PBS or 1x10^{11} vg AAV6.2-CMV-GFP. Micrographs were taken at 5x magnification. Representative images of n= 5 animals per group are shown.

In summary, the results demonstrate that AAV6.2 is a potent vector for the efficient transduction of airway epithelial and type II alveolar epithelial cells in mice. Moreover, no acute inflammation following vector administration could be detected, which enabled long-term gene expression for at least 4 months after a single vector application.
2.2 Modeling pulmonary fibrosis by AAV6.2-mediated TGFβ1 expression

2.2.1 Construct design and validation of TGFβ1 bioactivity

After having proven AAV6.2’s capability to efficiently transduce murine lung cells in vivo, it was next investigated whether the degree of overexpression is sufficient to induce phenotypic changes. As a proof-of-concept approach, AAV6.2 was used to express TGFβ1 in the lung of mice, because it is well established that TGFβ1 overexpression induces pulmonary fibrosis in rodents. For this purpose, a codon-usage-optimized full-length cDNA of the coding sequence of murine TGFβ1 was ordered and cloned into a pAAV vector harboring AAV2 ITRs flanking an expression cassette that contains a CMV promoter and hGH polyA sequence (for plasmid map, see Figure 40b). The TGFβ1 gene also contained C223S and C225S mutations which destabilize the clamp-like structure of the LAP domain, thereby promoting the release of mature, active TGFβ1 protein (202). Using the pAAV-CMV-TGFβ1 construct, AAV6.2-CMV-TGFβ1 vectors were produced and tested for bioactivity. To this end, the lung epithelial cell line NCI-H292 and normal human lung fibroblasts (NHLF) were transduced with increasing amounts of AAV6.2- TGFβ1. As expected, secreted TGFβ1 protein was detected in the cell culture supernatant of both cultures, which correlated well with TGFβ1 gene expression levels that were measured by qPCR (Figure 10). Intact TGFβ1 downstream signaling was evident from a largely vector dose-dependent increase in Plasminogen activator inhibitor (PAI-1) gene expression, a prominent TGFβ1 downstream target and surrogate marker (203) for TGFβ1 activity (Figure 10).

Figure 10: Validation of AAV6.2-mediated TGFβ1 bioactivity in NCI-H292 and NHLF cells

(a) NCI-H292 and (b) NHLF cells were transduced with increasing concentrations of AAV6.2-CMV-TGFβ1. 72 h after transduction, TGFβ1 gene expression and protein secretion were measured by qPCR and ELISA, respectively. Gene expression of PAI-1 was measured by qPCR as a surrogate marker of TGFβ1 downstream signaling. n=2. Mean +/- SD. A.U. = arbitrary units. NHLF= normal human lung fibroblast.
Results

To further validate construct integrity, the lung epithelial cell line A549 was transfected with the pAAV-CMV-TGFβ1 plasmid or a GFP control construct and incubated for six days. In accordance with the expectations for TGFβ1 signaling in these cells, epithelial-to-mesenchymal transition (EMT) was observed, as evident from the change to a spindle-shaped morphology in TGFβ1- but not GFP-expressing cells (Figure 11a).

Besides EMT, fibroblast-to-myofibroblast transition (FMT), which can also be induced in vitro by TGFβ1, is regarded as an important aspect contributing to the pathology of fibrosis. Therefore, it was further assessed whether TGFβ1 expressed upon transduction with the AAV6.2-CMV-TGFβ1 vectors would induce FMT in normal human lung fibroblasts (NHLF). For this purpose, NHLF cells were stimulated with the TGFβ1-containing cell supernatants derived from cells previously transduced with increasing doses of AAV6.2-CMV-TGFβ1 (see Figure 10). Three days after addition of conditioned medium (CM), the NHLF cells were fixed and immunostained for α-smooth muscle actin (αSMA), a cellular marker for myofibroblasts. As evident from fluorescence microscopy, the number of αSMA fibers was strongly increased in a TGFβ1 dose-dependent fashion, independent of whether TGFβ1-containing media was derived from transduced NCI-H292 or NHLF cells, whereas control medium did not induce any changes (Figure 11b). The dose-dependent increase in αSMA was further confirmed by automated, quantitative image-analysis, which calculates the number of αSMA fibrils per cell (where the number of cells is defined by the number of Hoechst 33342-stained nuclei) (Figure 11c).

Taken together, the results demonstrate that the AAV6.2-CMV-TGFβ1 vectors express functional TGFβ1 protein that is capable of inducing expected fibrosis-associated effects (EMT and FMT).
Figure 11: Validation of TGFβ1 bioactivity in EMT and FMT assays

(a) A549 cells grown in collagen-coated plates were transfected with either a GFP- or TGFβ1-expressing plasmid and incubated under serum-reduced (1% FCS) conditions for six days. Cell morphology was assessed by microscopy at 10x magnification. Representative images of n= 4 replicates are shown.

(b) NHLF cells were starved in serum-free medium for 24 h and subsequently treated with recombinant TGFβ1 (5 ng/mL) or conditioned medium (CM) derived from either NHLF or NCI-H292 cells that had previously been transduced with increasing concentrations of AAV6.2-CMV-TGFβ1 (see Figure 10). 72 h after treatment, nuclei were stained with Hoechst 33342 (blue) and α-smooth muscle actin (αSMA) fibers were stained with an AlexaFluor647-coupled antibody (red). αSMA fibers were assessed by (b) fluorescence microscopy and (c) quantified by automated image analysis. n=4. Mean +/- SD.
2.2.2 TGFβ1-induced pathophenotype (dose-response-relationship)

After having validated the functionality of the AAV6.2-CMV-TGFβ1 vectors in vitro, it was assessed whether the vector induces TGFβ1 expression in vivo and whether overexpression of this cytokine triggers fibrotic changes in the lung of treated animals, as expected based on previous Adenovirus vector-based studies (102)(144). To this end, increasing doses of AAV6.2-CMV-TGFβ1 or AAV6.2-stuffer vectors were applied to the lung of mice by intratracheal administration. To monitor the overall health status of the animals, body weight was recorded every other day. The body weight curves showed that mice that had received high-dose TGFβ1 vector – unlike control-treated animals that normally gained in weight – started to continuously lose weight from about one week after AAV application (Figure 12), indicating disease development.

![Figure 12: Body weight of mice following administration of increasing doses of AAV6.2-CMV-TGFβ1](image)

Mice received 0.3x10^{11}, 0.9x10^{11} or 2.7x10^{11} vg of either AAV6.2-stuffer or AAV6.2-CMV-TGFβ1 by i.t. application and body weight was monitored over the full experimental duration of 3 weeks. n=3 (PBS) and n=5 (AAV) animals per group. Mean +/- SEM. ***p<0.001, relative to AAV-stuffer 2.7.

Three weeks after AAV administration, the mice were euthanized and the lungs were flushed to receive bronchoalveolar lavage (BAL) samples, before being processed for RNA isolation and histological examination. Analysis of BAL TGFβ1 protein levels showed an AAV-dose dependent increase of TGFβ1 that occurred specifically for AAV6.2-CMV-TGFβ1 but not with AAV-stuffer (Figure 13). Notably, only a small proportion of TGFβ1 was found to be present in its activated form. When measuring gene expression of the TGFβ1 downstream target PAI-1, the fibrosis markers Collagen type 1 α 1 (COL1A1), Matrix metalloproteinase 2 (MMP2), Tissue inhibitor of MMPs 1 (TIMP1) as well as the Marker of proliferation KI-67, a TGFβ1-specific and mostly dose-dependent increase was observed (Figure 13).
Results

Figure 13: Analysis of AAV6.2-CMV-TGFβ1-mediated gene expression changes in the lung of mice

Mice received 0.3x10^{11}, 0.9x10^{11} or 2.7x10^{11} vg of either AAV6.2-stuffer or AAV6.2-CMV-TGFβ1 by i.t. administration and 21 days later, TGFβ1 levels were measured in the BAL of mice using ELISA. Gene expression levels of Plasminogen activator inhibitor 1 (Pai-1), Collagen type 1 α 1 (Col1a1), Matrix metalloproteinase 2 (Mmp2), Tissue inhibitor of metalloproteinases 1 (Timp1) and Marker of proliferation Ki-67 were assessed by qPCR using total lung RNA. n =3 (PBS) and n=5 (AAV) animals per group. Mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001.

To investigate whether AAV-mediated TGFβ1 overexpression indeed induced fibrotic changes evident from altered tissue morphology, the left lung of each animal was processed to FFPE sections and fibrosis was assessed histopathologically by Masson trichrome staining. This staining results in dark red to pink nuclei, light red cytoplasm and blue collagen. When examining whole lung sections at low magnification, relatively widespread fibrosis was observed in AAV-CMV-TGFβ1- but not AAV-stuffer-treated animals (Figure 14, left panel). Closer examination revealed considerable fibrosis characterized by alveolar septa thickening, immune cell influx and increased deposition of connective tissue, as shown in detail in Figure 14 (right panel). Scoring of disease severity by a histopathologist according to the criteria defined by Ashcroft (204) resulted in a mean fibrosis score of 3.2 in the high-dose AAV-CMV-TGFβ1 group. Whereas in human lung biopsies an Ashcroft score of 8 indicates maximal fibrotic damage, in mice – where whole lung sections are being analyzed – an Ashcroft score of approximately 4 can be regarded as the maximal possible value, due to often lethal health impairment at this degree of tissue damage.
Results

Figure 14: Histological assessment of AAV6.2-CMV-TGFβ1-induced pulmonary fibrosis
Masson trichrome-stained FFPE sections of the left lung lobe of mice, 21 days after i.t. administration of either $2.7 \times 10^{11}$ vg AAV-CMV-TGFβ1 or AAV-stuffer control vector. Selected areas are presented magnified and are referred to in the whole section as boxes. The right panel of micrographs represents 5x magnified details of the middle panel. Scale bars: 5 mm and 200 µm. White arrowheads: thickened alveolar septum. Black arrowheads: macrophages. White asterisk: area of massive collagen deposition (blue-stained fibrils). Representative images of n=5 animals per group are shown.

The presence of macrophages, neutrophils and lymphocytes was also confirmed by analyzing BAL samples using a hematology analyzer (Figure 15a). As an indirect evidence of epithelial damage and vascular leakage, the amount of total protein in BAL samples was also strongly increased in TGFβ1-overexpressing animals (Figure 15b). Moreover, indicative of structural remodeling and collagen deposition, a massive, AAV-CMV-TGFβ1 dose-dependent increase in lung weight was observed in fibrotic animals (Figure 15c).
Figure 15: Analysis of immune cells, BAL protein and lung weight following TGFβ1 overexpression

Analysis of (a) immune cells present in BAL samples, (b) total BAL protein and (c) wet lung weight 21 days after i.t. administration of $0.3 \times 10^{11}$, $0.9 \times 10^{11}$ or $2.7 \times 10^{11}$ vg of either AAV-CMV-TGFβ1 or AAV-stuffer control vector to the lung of mice. n=3 (PBS) and n=5 (AAV) animals per group. Mean +/- SEM. ***p<0.001, relative to AAV-stuffer 2.7.

Besides histological analysis of lung biopsy samples, abnormalities identified by computed tomography (CT) and shortness of breath resulting from reduced lung compliance are important signs and symptoms that are taken into account for clinical diagnosis of pulmonary fibrosis. Notably, pulmonary fibrosis induced by AAV-mediated TGFβ1 overexpression was clearly evident from micro-CT analysis (Figure 16a) and also resulted in strongly impaired lung function (Figure 16b), thereby reflecting clinically relevant symptoms.

Figure 16: Micro-CT and lung function analysis of mice after AAV-CMV-TGFβ1-induced fibrosis

(a) Micro-computed tomography (CT) and (b) assessment of lung function (compliance and forced vital capacity (FVC)) in mice, 21 days after i.t. administration of $0.3 \times 10^{11}$, $0.9 \times 10^{11}$ or $2.7 \times 10^{11}$ vg of either AAV-CMV-TGFβ1 or AAV-stuffer control vector. In (a) representative images of animals that had received high-dose vector are shown. n=3 (PBS) and n=5 (AAV) animals per group. Mean +/- SEM. *p<0.05, ***p<0.001, relative to respective AAV-stuffer controls.
2.2.3 AAV-TGFβ1- vs. Bleomycin-induced fibrosis: Comparative analysis of the disease course and gene expression profiles

After having identified an AAV dose suitable to induce pulmonary fibrosis, thereby mirroring critical disease features, we next aimed at investigating disease development and progression over time. Comprehensive characterization of the disease course will not only help to define time points for compound application and readouts for use of the model in pharmacological studies, but also help to identify regulators and pathways central to critical disease stages such as acute onset/fibrogenesis and fibrosis maintenance. In order to identify potential differences of the novel AAV-TGFβ1-based model and the well-established and widely used single-application bleomycin-induced model, both models were studied in parallel for a period of 4 weeks. For this purpose, mice either received 1 mg/kg Bleomycin or 2.5x10^{11} vg AAV6.2-CMV-TGFβ1 by intratracheal administration and fibrosis was assessed 3, 7, 14, 21 and 28 days post treatment. Additionally, at each time point, total lung RNA was prepared from whole lung homogenates and analyzed by next generation sequencing to obtain mRNA and miRNA expression profiles.

2.2.3.1 Fibrosis phenotype

As observed in the previous experiment, AAV6.2-CMV-TGFβ1-treated animals started to continuously lose weight from approximately 1 week after AAV administration, reaching an average weight loss of 20 % by 4 weeks (Figure 17a).

Figure 17: Body weight of mice following application of either AAV-TGFβ1 or Bleomycin

Mice either received NaCl, 1 mg/kg Bleomycin or 2.5x10^{11} vg of either AAV6.2-stuffer or AAV6.2-CMV-TGFβ1 by i.t. administration and body weight was monitored over the full experimental duration of 4 weeks. (a) mean body weight for each group. (b) body weight of single animals. n=36 (NaCl), n=48 (Bleomycin) and n=30 (AAV) initially treated animals less 6 (NaCl), 8 (Bleomycin) and 5 (AAV) animals, respectively, at each time point. Mean +/- SEM.
Similarly, mice that had received Bleomycin acutely lost weight beginning at day 3 after application, whereas both control groups (NaCl control for Bleomycin-treatment, AAV-stuffer control for AAV-TGFβ1 treatment) normally gained in weight. However, unlike in the AAV group, the body weight course of single Bleomycin-treated animals appeared more heterogeneous and some of the mice started to recover after initially losing weight (Figure 17b).

It is conceivable that the difference between the acute (Bleomycin) and timely shifted (AAV-TGFβ1) disease onset is due to the different modes of disease induction, i.e. acute injury (Bleomycin) vs. TGFβ1 overexpression. In fact, several results underscore this assumption. For instance, the shift was independent of the fact that even at the earliest time point (day 3), TGFβ1 levels in the AAV model strongly exceeded the endogenously present TGFβ1 levels triggered by Bleomycin (Figure 18), pointing towards the direction that acute injury rather than initial TGFβ1 induction is crucial for the sudden onset of disease in the Bleomycin model. Furthermore, immune cells, in particular neutrophils, were specifically increased at day 3 following bleomycin administration and only occurred in the AAV model from day 14 onwards (Figure 19). These findings were in accordance with the expression pattern of the neutrophil-attracting cytokine KC/Cxcl1. A similar pattern was also seen for monocytes and lymphocytes (Figure 19). In this case, too, the levels of IL12, which is secreted by activated macrophages and dendritic cells, were increased in a similar way (Figure 19 inset). Likewise, this temporal shift was also observed in most other measurements, e.g. lung weight, total BAL protein and the decrease in lung function (Figure 20). Of note, however, is that despite the difference in disease onset, the phenotype in both models was very similar at day 21 in all readouts analyzed (Figure 17a, Figure 19, Figure 20).

![Figure 18: TGFβ1 BAL levels over time following administration of either AAV-TGFβ1 or Bleomycin](image)

Total TGFβ1 protein levels were determined by ELISA in BAL samples at every indicated time point after administration of either Bleomycin or AAV-TGFβ1 (see details in Figure 17). n=6 (NaCl), n=8 (Bleomycin) and n=5 (AAV) animals per group. Mean +/- SEM. **p<0.01, ***p<0.001. ns= not significant.
Figure 19: Differential immune cell counts following application of either AAV-TGFβ1 or Bleomycin

Total and differential immune cell counts were determined in BAL samples at every indicated time point after administration of either Bleomycin or AAV-TGFβ1 (see details in Figure 17). Insets: IL12 [ng/mL] and KC (CXCL1) [pg/mL] protein levels measured in BAL samples using ELISA. n=6 (NaCl), n=8 (Bleomycin) and n=5 (AAV) animals per group. Mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001. ns= not significant.

Figure 20: Lung weight, BAL protein and lung function upon AAV-TGFβ1 or Bleomycin application

(a) Wet lung weight, (b) total BAL protein, (c) lung compliance and (d) forced vital capacity (FVC) were assessed at indicated time points after administration of either Bleomycin or AAV-TGFβ1 (see details in Figure 17). n=6 (NaCl), n=8 (Bleomycin) and n=5 (AAV) animals per group. Mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001. ns= not significant.
Results

2.2.3.2 Gene expression profile

In order to dissect the molecular pathways and overall changes in gene expression underlying disease development and progression in the two models of pulmonary fibrosis, RNA was prepared from total lung homogenates of each animal and applied to next generation sequencing (NGS) analysis. Quality control analysis of NGS raw data showed that over 20 million reads were reached for all of the samples sequenced. Due to the use of a sequencing library preparation kit that does not specifically select for mRNAs (e.g. by binding of the polyA tail), but uses all RNA present in a sample, exonic reads only made up 34 %, whereas intronic, intergenic and other reads represented 66 % of all reads.

Initially, hierarchical clustering was performed using the union of all genes that were significantly altered (here: adjusted p-value <0.001) at at least two time points to identify the degree of similarity among the gene expression patterns of the different experimental groups and time points (Figure 21). Besides showing that the early changes in the Bleomycin model (day 3) cluster together with the time points, where the first obvious phenotypic changes in the AAV model were observed (day 7 and 14), the analysis confirmed high similarity between both models on day 21, thereby reflecting previous phenotypic observations.

![Figure 21: Hierarchical clustering of the different experimental groups](image-url)

Hierarchical clustering of gene expression data derived from RNA-sequencing analysis of total lung RNA obtained from mice treated with either AAV-TGFβ1 or Bleomycin, as described in detail above (e.g. Figure 17). Clustering was performed using the union of all genes with an adjusted p-value of < 0.001 at at least two time points independent of the model. Green= downregulated genes. Red= upregulated genes.
After re-sorting the data by the disease model and time points, two gene clusters with a timely differential expression pattern became obvious among the upregulated genes: Genes in cluster 1 (Figure 22) were exclusively and strongly deregulated at day 3 and 7 in the Bleomycin model and only weakly altered from day 7 in the TGFβ1 model, whereas genes of cluster 2 were upregulated in later phases of both models. Notably, when the clusters of genes were analyzed for enriched pathways, the genes in cluster 1 were found to be associated with processes of wounding, defense response and cytokine signaling, whereas for cluster 2 genes an enrichment of fibrotic processes such as extracellular matrix (ECM) receptor interaction, focal adhesion and collagen fibril organization was observed. Thus, these findings illustrate that the previous phenotypic observations are well reflected by the gene expression changes measured using NGS.

Figure 22: Pathway analysis of gene clusters with temporally differentiated expression patterns

Gene clusters were defined based on their different temporal expression patterns. Cluster 1: early upregulated genes; Cluster 2: late upregulated genes. The respective lists of genes were applied to pathway enrichment analysis using EnrichR and the three most significantly enriched KEGG pathways and gene ontology (GO) biological processes are listed for each cluster.

To further investigate similarity of the models, the number of differentially expressed genes (defined as those genes with a log2-fold-change $<-0.6$ or $>0.6$ and an adjusted p-value $<0.05$) that were exclusively deregulated in one or commonly deregulated in both models was calculated (Figure 23a). Both, the time course of the total number of differentially expressed genes (Figure 23b) and the analysis of common genes confirmed that there is little overlap at the early time points (day 3 and 7), whereas a large overlap (47 %) was evident at day 21 (Figure 23a). Of note is
that the commonly deregulated genes show a very high correlation with regard to their direction and extent of deregulation (Figure 23a, lower panel). Interestingly, when the fractions of exclusive or common genes at day 21 were analyzed for enriched pathways, processes associated with inflammation, defense and wounding responses were enriched for Bleomycin-exclusive genes, whereas enrichment for cell cycle-associated processes was found for TGFβ1-exclusive genes. As expected, for the fraction of common genes, fibrosis-associated processes were identified (Figure 23c). To further investigate the genes exclusively altered in one or the other model, lists of genes that were significantly expressed at any of the five time points in both models were overlaid and separated for exclusive and common genes. Using this strategy, 1315 AAV-exclusive, 1230 Bleomycin-exclusive and 2719 common genes were identified. While no additional insight was received for AAV-exclusive and common genes, pathway analysis using the Bleomycin-exclusive genes revealed Toll-like receptor- and interferon-signaling as being significantly enriched.

Figure 23: Exclusively and commonly deregulated genes of the Bleomycin and AAV-TGFβ1 models

For each time point, the number of significantly deregulated genes (AAV-TGFβ1 vs. AAV-stuffer and Bleomycin vs. NaCl treatment) was calculated, where following criteria were applied: log2-fold-change < -0.6 or >0.6, adjusted p-value < 0.05. (a) Out of the resulting lists of genes, the fraction of genes exclusively expressed in one of the models or commonly expressed in both models was calculated (pie charts). The lower part of the graph shows correlation plots for the commonly deregulated genes and the Pearson correlation coefficient r is depicted in the upper left corner of each graph. (b) Number of differentially expressed genes over time (AAV-TGFβ1 vs. AAV-stuffer, Bleomycin vs. NaCl). (c) Pathway enrichment analysis using the exclusively and commonly deregulated genes of day 21. The three most significant GO biological processes enriched for each of the gene lists are depicted. log2fc= log2-fold change.
Results

Reactome pathway analysis further demonstrated that processes linked to inflammation (“Cytokine signaling in immune system”) and wounding (“Formation of Fibrin Clot (Clotting Cascade)”) were specifically active in the Bleomycin model at the early time points (Figure 24). Interestingly, despite the absence of initial lung injury in the AAV-TGFβ1 model, coagulation became evident during the highly fibrotic phases in the disease model at day 21 and 28 (Figure 24). As previously observed, the earlier onset of fibrosis in the Bleomycin model was reflected by an initially higher enrichment for extracellular matrix- (ECM) and collagen synthesis-associated processes, which, however, was compensated in the AAV-TGFβ1 model after the rapid development of fibrosis from day 14 onwards (Figure 24).

Figure 24: Enrichment analysis of wounding- and fibrosis-associated pathways over time

For each time point and experimental group (Bleomycin or AAV-TGFβ1 treatment, see above), lists of differentially expressed genes (log2-fold-change < -0.6 or >0.6, adjusted p-value <0.05) were analyzed for enriched pathways by Reactome pathway analysis using EnrichR. For selected pathways, p-values were extracted and plotted over time. ECM= extracellular matrix.

In order to further deepen our understanding of mechanistic differences between the two models, upstream regulator analysis was performed using Ingenuity pathway analysis (IPA). Based on the gene expression data and known protein-protein/transcription factor interaction networks, IPA calculates the probability for potential upstream regulators to be responsible for the observed changes in gene expression. Similarly, the IPA downstream analysis tool explores the enrichment of potential downstream functions and processes.
Results

The data again show that at the early time points in the Bleomycin model, inflammation-associated regulators such as TNFα, IL6 and an inhibitor of NFκB (NFKBIA) are strongly enriched, as are regulators of DNA damage/apoptosis (p53) and coagulation (thrombin/F2) (Figure 25a). In contrast, in the AAV-TGFβ1 model, enrichment for these regulators was increasingly observed at later time points during the phases of onset and maintenance of fibrosis (days 7-28). As expected, TGFβ1 regulation was found to be the top enriched (p = 10\(^{-98}\)) regulator in both disease models during the phase of fibrosis maintenance (days 21 and 28). Pro-fibrogenic PDGF-BB was enriched in a similar way in both models with the timely shift observed before, as was the fibroblast growth factor FGF-2. Enrichment was further observed for IL-13, a potentially pro-fibrotic TH2 cytokine and the Wnt downstream target and hyaluronic acid receptor CD44, which is implicated in cell-adhesion and migration via interaction with MMPs, collagens and osteopontin. In line with the observed enrichment of angiogenesis-associated regulators at the late time points in both models, HIF-1α also showed particularly high enrichment during this disease stage. Much in accordance with the upstream regulator data, the temporally differentiated enrichment of cell proliferation and movement-, wounding-, cell-death- and fibrosis-associated processes further confirmed the previously observed phenotype of both models (Figure 25b).

Figure 25: Enrichment analysis for potential upstream regulators and downstream functions

For each experimental group (Bleomycin or AAV-TGFβ1 treatment, see above), differential gene expression (log2-fold-change < -0.6 or >0.6, adjusted p-value < 0.05) was analyzed over time using the Ingenuity Pathway Analysis (IPA) comparison analysis tool. (a) Based on the observed gene expression changes and known protein-protein/transcription factor interaction networks, the activity of potential upstream regulators that could explain the observed gene expression changes was calculated. The p-values for the enrichment of selected upstream regulators that were predicted to be active, were plotted over time. (b) Analysis of downstream functions. The enrichment of selected functions and processes is depicted by p-values plotted over time.
In an attempt to characterize the different disease stages (early, intermediate, late) in more detail, the gene expression data were filtered for genes with specific expression profiles, i.e. selectively high expression during the early/acute phase (day 3), the intermediate phase of fibrosis onset (day 7/14) and the late (day 21) experimental stage of fibrosis maintenance/progression. To select for genes with such specific expression profiles, defined filter criteria were applied, which are depicted in the legend of Figure 26. Each set of genes was then analyzed for enriched GO biological pathways to identify the most prominent processes of each disease stage.

The analysis revealed that the inflammatory reaction upon Bleomycin treatment was strongly associated with interferon signaling, as evident from the presence of five interferon-related genes among the top ten upregulated genes (Figure 26a) and a highly significant ($p= 9.9 \times 10^{-17}$) enrichment of the type I interferon signaling pathway. Identification of a cytokine stimulus-associated pathway further confirms the inflammatory phenotype observed at this acute disease stage. For the AAV-TGFβ1 model, only 21 genes had an expression profile compliant with the filter criteria, which is why no significantly enriched processes were identified and no data is shown for this time point.

In the intermediate phase (i.e. transition from an inflammatory to fibrotic phenotype in the Bleomycin model and onset of fibrosis in the AAV-TGFβ1 model), both models were characterized by gene expression changes associated with proliferation and cell division (chromosome segregation, cytokinesis, mitotic cell cycle) (Figure 26b and d). Interestingly, when analyzing the set of downregulated genes of the AAV model, pathway analysis identified “negative regulation of epithelial cell proliferation” as a significantly altered process. Notably, epithelial proliferation/hyperplasia and remodeling are key characteristics of pulmonary fibrosis.

As expected for the set of genes specifically upregulated during the fibrotic phase of both models (day 21), the most significantly enriched pathway was “extracellular matrix organization” (Figure 26c and e). Of note is that in the Bleomycin model, most of the top twenty upregulated genes encoded immunoglobulin domains, thereby indicating a strong antibody response at this disease phase. Hence, in addition to the top ten, the ten most upregulated non-immunoglobulin genes are also shown in Figure 26c. Interestingly, CTHRC1, a potential biomarker for IPF (205), represented the top hit in this set of genes. Moreover, many fibrosis-related genes – among them osteopontin and thrombospondin 2 – were part of the list of most distinctly upregulated genes in the AAV model (Figure 26e). Finally, “surfactant homeostasis” was found enriched among the downregulated genes in the AAV model, thereby indicating impaired alveolar function. In fact, at the late time points, the expression of the surfactant proteins A, B and C was significantly decreased in both models (data not shown).
Results

Both, Bleomycin (a-c) and AAV-TGFβ1 (d, e) gene expression data sets were filtered for genes with specific expression profiles, i.e. (a) early, (b, d) intermediate and (c, e) late differential expression, using following filter criteria: “Early”: significant deregulation (log2 fold change >0.6 or <0.6, q < 0.05) on day 3; no significant dereg. on day 21 and 28 (log2fc <0.6 and >0.6); focus on prominent deregulation on day 3 and 7 (d7/d14 log2fc ratio >1), “Intermediate”: significant dereg. on day 7 and 14 (Bleomycin) and day 14 (AAV-TGFβ1), respectively; no dereg. on day 3 and 28; focus on prominent deregulation on day 14 (d14/d21 log2fc ratio >1), “Late”: significant dereg. on day 21; no dereg. on day 3 and 7; focus on prominent deregulation on day 21 (d14/d21 log2fc ration <1). The resulting lists of up- and downregulated genes were separately applied to GO biological process enrichment analysis using EnrichR. The top ten deregulated genes are shown for each panel along with their expression profile over time (3, 7, 14, 21, 28 days after application) and a selection of significantly enriched pathways. Downregulated genes are only shown when meaningful pathways were found to be enriched for the respective genes (d, e). Because in (c) all top ten genes encoded immunoglobulins, the top ten non-immunoglobulin genes were additionally included.

Figure 26: Analysis of early, intermediate and late gene expression changes
Results

Taken together, the analysis suggests that the main difference between the two models lies in the acute injury by Bleomycin and associated early inflammation and wounding responses, whereas TGFβ1 seems to trigger fibrotic changes along with rather simultaneously occurring inflammation.

Given that, despite the different modes of disease induction (Bleomycin-mediated lung injury vs. transgenic overexpression of a key disease-driving gene) a big phenotypic and molecular overlap at the fibrotic disease stage was observed, it is reasonable to assume that certain mechanisms are mandatory for fibrogenesis and disease maintenance. To identify the genes likely involved in these central mechanisms, the list of differentially expressed genes on day 21 in the AAV-TGFβ1 model was overlaid with the genes deregulated on day 14 and/or 21 in the Bleomycin model. Filtering for those genes that were commonly expressed in both models and that showed the same direction (up/downregulated) of expression (average of the five time points), resulted in a list of 1875 commonly deregulated genes, 1120 of which were up- and 755 of which were downregulated. The top 110 upregulated and top 50 downregulated genes along with their gene expression profiles over time are shown in Table 2 and Table 3, respectively. EnrichR protein-protein-interaction analysis using all 1875 genes revealed non-receptor tyrosine kinase SRC, integrin beta 1 (ITGB1), protein kinase C α and β (PRKCA, PRKCB), epidermal growth factor receptor (EGFR), and the SRC kinase family members LYN and FYN as the top interaction partners (p-value range: 1.1x10^{-10}-6.7x10^{-13}).
### Results

Table 2: Commonly upregulated genes in the fibrotic phase of the Bleomycin and AAV-TGFβ1 models

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene</th>
<th>Description</th>
<th>Location/Type</th>
<th>Expression profile</th>
<th>log2Fc (day 21)</th>
<th>FPKM (day 21)</th>
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<tbody>
<tr>
<td>1</td>
<td>Col11a1</td>
<td>Collagen alpha-1(XII) chain</td>
<td>Secreted</td>
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<td>6.12</td>
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<td>2</td>
<td>Saa3</td>
<td>Serum amyloid A 3</td>
<td>Secreted</td>
<td>6.21</td>
<td>5.35</td>
<td>177.05</td>
</tr>
<tr>
<td>3</td>
<td>Chi1</td>
<td>Neural cell adhesion molecule L1-like protein</td>
<td>Secreted</td>
<td>6.41</td>
<td>5.45</td>
<td>14.42</td>
</tr>
<tr>
<td>4</td>
<td>Ereg</td>
<td>Proepiregulin</td>
<td>Secreted</td>
<td>5.43</td>
<td>4.95</td>
<td>5.63</td>
</tr>
<tr>
<td>5</td>
<td>Timp1</td>
<td>Metalloproteinase inhibitor 1</td>
<td>Secreted</td>
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<td>4.11</td>
<td>266.92</td>
</tr>
<tr>
<td>6</td>
<td>Darc</td>
<td>Atrial chemokine receptor 1</td>
<td>Transmembr.</td>
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<td>4.88</td>
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<tr>
<td>7</td>
<td>Spp1</td>
<td>Osteopontin</td>
<td>Secreted</td>
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<td>3.97</td>
<td>3506.02</td>
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<td>8</td>
<td>Frzb</td>
<td>Secreted frizzled-related protein 3</td>
<td>Secreted</td>
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<td>6.04</td>
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<td>9</td>
<td>Grem1</td>
<td>Gremlin-1</td>
<td>Secreted</td>
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<td>5.05</td>
<td>7.27</td>
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</table>

**Note:**
- **AAV:** Adenovirus-associated virus
- **Bleo:** Bleomycin
- **FPKM:** Fragments Per Kilobase of exonic sequence per million mapped reads

**Expression profile log2fc (day 21):**
- **AAV:** Adenovirus-associated virus
- **Bleo:** Bleomycin
Continuation of Table 2

<table>
<thead>
<tr>
<th>Rank</th>
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<th>Description</th>
<th>Location/Type</th>
<th>AAV log2fc (day 21)</th>
<th>Bleo log2fc (day 21)</th>
<th>FPKM log2fc (day 21)</th>
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<td>Protein FAM167B</td>
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<td>7.93</td>
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<td>122</td>
<td>Fkbp5</td>
<td>Peptidyl-prolyl cis-trans isomerase FKBP5</td>
<td>other Enzyme</td>
<td>3.29</td>
<td>1.16</td>
<td>52.89</td>
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<td>8.84</td>
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</table>

Table 2 shows the top 110 non-immunoglobulin genes commonly upregulated during the fibrotic phase of the Bleomycin- and AAV-TGFβ1-induced models of fibrosis. Of all genes significantly deregulated (log2-fold change (log2fc) >0.6 or < -0.6 and FDR-adjusted p-value (q) <0.05) on day 14 and/or day 21 in the Bleomycin model (relative to NaCl) and day 21 in the AAV-TGFβ1 model (relative to AAV-stuffer), those showing overall similar expression (up/downregulation), as assessed by comparing the mean log2fc expression values over all time points, were included in the list. The genes were sorted by the mean log2fc, which was calculated as mean value of the average log2fc expression in the Bleomycin model (day 14 + day 21) and the log2fc expression at day 21 in the AAV-TGFβ1 model. The expression profiles depict individually scaled log2fc changes over all experimental time points (3, 7, 14, 21, 28 days). 23 genes were immunoglobulin-domain-encoding and were excluded from this table. TF= transcription factor. Transmembr.= transmembrane protein. FPKM values (fragments per kilobase of exon per million fragments mapped) indicate absolute gene expression under diseased (i.e. AAV-TGFβ1- and Bleomycin-treated) conditions.
Table 3: Commonly downregulated genes in the fibrotic phase of the Bleomycin and AAV-TGFβ1 models

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene</th>
<th>Description</th>
<th>Location/Type</th>
<th>Expression profile</th>
<th>log2fc (day 21)</th>
<th>FPKM (day 21)</th>
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<tbody>
<tr>
<td>1825</td>
<td>Slc4a5</td>
<td>Electrogenic sodium bicarbonate cotransporter 4</td>
<td>Transporter</td>
<td>AAV Bleo AAV Bleo AAV Bleo</td>
<td>-2.31 -1.69</td>
<td>2.42 5.15</td>
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<td>Gens1e</td>
<td>Liver carboxylesterase 1</td>
<td>other Enzyme</td>
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<td>-2.57 -1.21</td>
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<td>Synaptotagmin-17</td>
<td>Transmembr.</td>
<td></td>
<td>-2.26 -2.05</td>
<td>0.30 0.44</td>
</tr>
<tr>
<td>1838</td>
<td>Pigs20s12</td>
<td>Prostaglandin-endoperoxide synthase 2, oppos. strand 2</td>
<td>unknown</td>
<td></td>
<td>-2.99 -1.13</td>
<td>0.29 0.67</td>
</tr>
<tr>
<td>1839</td>
<td>Angf5f8</td>
<td>Rho guanine nucleotide exchange factor 38</td>
<td>other</td>
<td></td>
<td>-2.28 -1.62</td>
<td>4.58 6.06</td>
</tr>
<tr>
<td>1840</td>
<td>N28178</td>
<td>Protein KIAA1045</td>
<td>Ubiquitome</td>
<td></td>
<td>-2.66 -1.63</td>
<td>0.78 1.65</td>
</tr>
<tr>
<td>1841</td>
<td>Knc2</td>
<td>Potassium voltage-gated channel subtype A member 2</td>
<td>Ubiquitome</td>
<td></td>
<td>-2.27 -2.33</td>
<td>0.67 0.54</td>
</tr>
<tr>
<td>1842</td>
<td>Ces1</td>
<td>Liver carboxylesterase 1</td>
<td>other Enzyme</td>
<td></td>
<td>-2.83 -1.29</td>
<td>0.78 2.71</td>
</tr>
<tr>
<td>1843</td>
<td>Qp6</td>
<td>Gap junction beta-6 protein</td>
<td>Transportembr.</td>
<td></td>
<td>-2.66 -1.17</td>
<td>0.57 1.28</td>
</tr>
<tr>
<td>1844</td>
<td>Mbo1</td>
<td>Mannose-binding lectin (protein A) 1</td>
<td>Secreted</td>
<td></td>
<td>-3.05 -0.68</td>
<td>0.14 0.56</td>
</tr>
<tr>
<td>1845</td>
<td>Shrult11</td>
<td>Small nuclear RNA host gene 11</td>
<td>other</td>
<td></td>
<td>-2.71 -1.36</td>
<td>0.71 1.59</td>
</tr>
<tr>
<td>1846</td>
<td>Cyg202</td>
<td>Cytorbrome P49.2F1</td>
<td>other Enzyme</td>
<td></td>
<td>-2.71 -1.46</td>
<td>88.31 256.56</td>
</tr>
<tr>
<td>1847</td>
<td>Cyg401</td>
<td>Cytorbrome P49.4B1</td>
<td>other Enzyme</td>
<td></td>
<td>-2.44 -1.99</td>
<td>56.11 73.87</td>
</tr>
<tr>
<td>1848</td>
<td>Cq2</td>
<td>Carbonyl reductase 2</td>
<td>other Enzyme</td>
<td></td>
<td>-2.75 -1.44</td>
<td>210.00 599.59</td>
</tr>
<tr>
<td>1849</td>
<td>Kira4</td>
<td>Killer cell lectin-like receptor, subfamily A, member 4</td>
<td>other</td>
<td></td>
<td>-2.71 -1.82</td>
<td>0.51 0.48</td>
</tr>
<tr>
<td>1850</td>
<td>Untm</td>
<td>Indolethylamine N-methyltransferase</td>
<td>other Enzyme</td>
<td></td>
<td>-2.05 -2.30</td>
<td>269.68 235.64</td>
</tr>
<tr>
<td>1851</td>
<td>Ta2r143</td>
<td>Taste receptor, type 2, member 143</td>
<td>unknown</td>
<td></td>
<td>-1.98 -3.10</td>
<td>0.47 0.14</td>
</tr>
<tr>
<td>1852</td>
<td>Adra1a</td>
<td>Alpha-1A adrenergic receptor</td>
<td>GPCR</td>
<td></td>
<td>-2.13 -2.25</td>
<td>2.94 1.89</td>
</tr>
<tr>
<td>1853</td>
<td>Gp9</td>
<td>Platelet glycoprotein IX</td>
<td>Transmembr.</td>
<td></td>
<td>-2.29 -2.13</td>
<td>0.79 1.16</td>
</tr>
<tr>
<td>1854</td>
<td>Sic7a10</td>
<td>Asc-type amino acid transporter 1</td>
<td>Transporter</td>
<td></td>
<td>-2.17 -2.46</td>
<td>3.43 3.16</td>
</tr>
<tr>
<td>1855</td>
<td>Aox3</td>
<td>Aldhyde oxidase 3</td>
<td>other Enzyme</td>
<td></td>
<td>-2.88 -1.56</td>
<td>8.26 17.79</td>
</tr>
<tr>
<td>1856</td>
<td>4930517o19Rik</td>
<td>RIKEN cDNA 4930517o19 gene</td>
<td>unknown</td>
<td></td>
<td>-1.85 -3.41</td>
<td>0.45 0.07</td>
</tr>
<tr>
<td>1857</td>
<td>Gm14964</td>
<td>Predicted gene 14964</td>
<td></td>
<td></td>
<td>-2.20 -2.57</td>
<td>1.41 1.65</td>
</tr>
<tr>
<td>1858</td>
<td>Ta2r135</td>
<td>Taste receptor type 2 member 60</td>
<td>Transportembr.</td>
<td></td>
<td>-2.17 -2.93</td>
<td>0.48 0.17</td>
</tr>
<tr>
<td>1859</td>
<td>Myh7</td>
<td>Myosin-7</td>
<td>other</td>
<td></td>
<td>-3.26 -1.56</td>
<td>0.36 1.23</td>
</tr>
<tr>
<td>1860</td>
<td>Asp2b2</td>
<td>Plasma membrane calcium-transporting ATPase 2</td>
<td>other Enzyme</td>
<td></td>
<td>-3.51 -1.50</td>
<td>0.14 0.90</td>
</tr>
<tr>
<td>1861</td>
<td>Nm1</td>
<td>Neuritin</td>
<td>Transmembr.</td>
<td></td>
<td>-2.92 -2.05</td>
<td>0.91 1.98</td>
</tr>
<tr>
<td>1862</td>
<td>4921513d19Rik</td>
<td>RIKEN cDNA 4921513d19 gene</td>
<td>unknown</td>
<td></td>
<td>-3.19 -1.79</td>
<td>0.40 0.60</td>
</tr>
<tr>
<td>1863</td>
<td>Gm13387</td>
<td>Predicted gene 13387</td>
<td>unknown</td>
<td></td>
<td>-3.36 -2.12</td>
<td>0.93 1.83</td>
</tr>
<tr>
<td>1864</td>
<td>Coper5</td>
<td>Copine-5</td>
<td>other</td>
<td></td>
<td>-2.75 -3.14</td>
<td>0.33 0.27</td>
</tr>
<tr>
<td>1865</td>
<td>Ppatp</td>
<td>Platelet basic protein</td>
<td>Secreted</td>
<td></td>
<td>-3.01 -2.63</td>
<td>7.44 6.33</td>
</tr>
<tr>
<td>1866</td>
<td>Tmemn132d</td>
<td>Transmembrane protein 132D</td>
<td>Transmembr.</td>
<td></td>
<td>-2.89 -2.64</td>
<td>0.23 0.31</td>
</tr>
<tr>
<td>1867</td>
<td>Kctd9</td>
<td>Keratin, type II cytoskeletal 76</td>
<td>other</td>
<td></td>
<td>-2.14 -3.51</td>
<td>1.02 0.42</td>
</tr>
<tr>
<td>1868</td>
<td>Gria1</td>
<td>Glutamate receptor 1</td>
<td>other</td>
<td></td>
<td>-2.77 -3.39</td>
<td>1.05 0.73</td>
</tr>
<tr>
<td>1869</td>
<td>Slc5a12</td>
<td>Sodium-coupled monocarboxylate transporter 2</td>
<td>Transporter</td>
<td></td>
<td>-3.42 -2.75</td>
<td>0.34 0.38</td>
</tr>
<tr>
<td>1870</td>
<td>Ihx2</td>
<td>Protein BEX1</td>
<td>other</td>
<td></td>
<td>-2.91 -3.11</td>
<td>1.54 1.52</td>
</tr>
<tr>
<td>1871</td>
<td>Cq7</td>
<td>Acetylcholinesterase collagenic tail peptide</td>
<td>other</td>
<td></td>
<td>-3.25 -3.22</td>
<td>0.67 0.73</td>
</tr>
<tr>
<td>1872</td>
<td>Acox1</td>
<td>Acyl-coenzyme A oxidase-like protein</td>
<td>other Enzyme</td>
<td></td>
<td>-4.21 -2.18</td>
<td>1.56 5.32</td>
</tr>
<tr>
<td>1873</td>
<td>Ank6d3</td>
<td>Ankyrin repeat domain-containing protein 63</td>
<td>other</td>
<td></td>
<td>-3.72 -2.95</td>
<td>0.23 0.49</td>
</tr>
<tr>
<td>1874</td>
<td>Hes2</td>
<td>Transcription factor HES-2</td>
<td>other</td>
<td></td>
<td>-4.26 -4.35</td>
<td>0.08 0.08</td>
</tr>
<tr>
<td>1875</td>
<td>Fapb1</td>
<td>Fatty acid-binding protein, liver</td>
<td>other</td>
<td></td>
<td>-5.02 -2.98</td>
<td>0.25 0.80</td>
</tr>
</tbody>
</table>

Table 3 shows the top 50 genes commonly downregulated during the fibrotic phase of the Bleomycin- and AAV-TGFβ1-induced models of fibrosis. The genes were identified using the criteria described in detail in Table 2. The expression profiles depict individually scaled log2fc changes over all experimental time points (3, 7, 14, 21, 28 days). TF= transcription factor. Transmembr.= transmembrane protein. FPKM values (fragments per kilobase of exon per million fragments mapped) indicate absolute gene expression levels under diseased (i.e. AAV-TGFβ1- and Bleomycin-treated) conditions.
In order to functionally classify the most distinctly upregulated genes, the top 200 non-immunoglobulin genes were analyzed for enriched biological processes. Out of those found enriched, certain biological processes of interest, including ECM organization, inflammatory response, angiogenesis and fibroblast proliferation were chosen and the genes contained in these processes were checked for multifunctional annotations. Out of the top 200, 71 genes were found in one or more of the selected processes and their functional annotations are shown in Figure 27.

![Figure 27: Functional classification of the top 200 commonly upregulated genes](image-url)

Besides the identification of many well-known genes with established functions in the context of inflammation and fibrosis, including osteopontin (SPP1), interleukin 6 (IL6), Chemokine (C-C motif) ligand 2 (CCL2), MMP2, fibronectin (FN1), elastin (ELN) and family members of the CTGF, PDGF, TGFβ and collagen family, also genes with little proposed involvement, much less an established mode of action in fibrosis, were identified. Among those, Lysyl oxidase-like 2 (LOXL2), Hyaluronan synthase 2 (HAS2) and Fibromodulin (FMODE) were found to be particularly interesting. LOXL2 is a
member of the lysyl oxidase family of collagen-crosslinking enzymes. Besides its role in ECM assembly, LOXL2 was also proposed to be involved in EMT induction (206). Hyaluronan (i.e. hyaluronic acid), the product of HAS2 enzymatic activity, is a glycosaminoglycan of the ECM, where it is involved in various processes during wound healing, cancer and inflammation, including cell migration, proliferation and attachment, particularly by interacting with its receptor CD44 (207)(208). The proteoglycan Fibromodulin might be involved in collagen fibril organization; it was shown to be necessary for fibroblast motility and efficient wound healing and at least partially required for the development of experimental liver fibrosis (209). Thus, the identification of these proteins builds a basis for further investigation of their role in pulmonary fibrosis and might suggest that antagonizing their function could be a possible strategy to modulate fibrosis development.

2.2.3.3 MicroRNA expression profile

In addition to gene expression profiling, which experienced rapid development with the advent of microarrays and next generation sequencing technology about fifteen to twenty years ago, profiling of small RNAs, including microRNAs (miRNAs), recently became a focus of extensive research. MicroRNAs are endogenously expressed, non-protein-coding small RNA molecules with an average length of 22 nucleotides and have been implicated as critical, post-transcriptional, multi-gene regulators of cellular processes in virtually every tissue of the body (210). MicroRNAs can individually target one mRNA, target several different mRNAs or cooperatively bind to different sites on the same mRNA target, thereby potentiating their repressive effects. Moreover, additional mechanisms such as miRNA-miRNA interactions are increasingly becoming apparent (211). Transcription of miRNA genes usually occurs by RNA polymerase II, resulting in hairpin-shaped pri-miRNAs that can be of several hundred base pairs in length and contain up to six different miRNA precursors (210). After being processed by the Drosha/DGCR8 complex, the resulting pre-miRNAs are exported to the cytoplasm by Exportin-5, where their hairpin loop is cut by Dicer, resulting in approximately 22 nucleotide long, miRNA-miRNA* duplex molecules. Mostly, only one of the opposite, not-perfectly-complementary miRNA strands is finally incorporated in the RNA-inducible silencing complex RISC, where binding between the miRNA and a target mRNA occurs. Target binding is usually only perfect in the “seed region” that comprises nucleotides 2-8. Repression of the target mRNA can either be achieved by degradation of the mRNA by Argonaute 2 proteins or by preventing translation, likely via steric hindrance/preventing the access of ribosomal machinery. With regard to nomenclature, miRNAs resulting from common precursors receive suffixes “a”, “b”, “c”, etc., while the suffixes “3p” and “5p” indicate the opposite strands (3’ and 5’) of the duplex miRNA.
In addition to their role as important regulators of gene expression and their use as disease biomarkers, miRNAs could also provide a way to delineate novel regulatory networks and co-regulated processes based on their interaction with (several) mRNA targets. Such networks might include regulatory loops, including negative feedback (i.e. gene y upregulation induces a mRNA y-targeting miRNA), incoherent feed-forward loops (gene y upregulation induces a second gene z along with a mRNA z-targeting miRNA) and coherent feed-forward loops (gene y upregulation suppresses a second gene z, which is additionally repressed by an upregulated gene z-specific miRNA) (212).

For the sequencing of miRNAs, RNA obtained from total lung homogenate was further processed to specifically enrich small RNAs (see 4.4.1 for details) and subsequently analyzed on a HiSeq 2000 sequencer.

224 and 232 miRNAs with significantly altered expression on day 21 (adjusted p-value <0.05) were found in the AAV-TGFβ1 and Bleomycin model, respectively, comprising a union of 290 different miRNAs. Of these, 166 miRNAs (= 57.2 %) were commonly deregulated in both models and 124 were exclusive for either one or the other model, confirming the big overlap seen before for the mRNA data. As expected, the common miRNAs showed a very high degree of correlation with regard to the direction (up/down) and strength of deregulation (Pearson correlation coefficient r=0.94). The top 20 up/downregulated miRNAs along with their expression profiles over time are shown in Table 4.

In an approach to directly correlate miRNA expression with functionally relevant fibrotic changes, the expression profiles of the miRNAs over time were analyzed for both correlation and anti-correlation with the decrease in lung function observed in the fibrosis models over time (see Figure 20). This strategy was based on the assumption that miRNAs, whose expression profile correlates with the lung function measurements (i.e. downregulation of miRNA in parallel to the decrease in lung function), could have mRNA targets that are potential drivers of the fibrotic response and whose translation is facilitated by the decreased expression of the miRNA. In turn, miRNAs that show strong anti-correlation (i.e. upregulation in parallel to the decrease in lung function) were hypothesized to have mRNA targets, whose suppression might promote fibrosis.
When the top anti-/correlated miRNAs were analyzed (Table 5), miR-199a-5p and miR-21a-5p were identified as the two top anti-correlated (r = -0.86 and -0.84, respectively) miRNAs, whereas miR-29a-3p was found reasonably correlated with the decrease in lung function (r = 0.67). Strikingly, miR-199a-5p and miR-21a-5p are among the most well-known miRNAs associated with...
fibrosis: Specifically, miR-199a-5p was shown to be overexpressed in fibrotic disorders of several organs, where it mediates Calveolin-1 suppression to increase TGFβ1 signaling and therefore promote fibrosis (213). Moreover, it was found significantly elevated in the serum of human IPF patients versus healthy controls (214). MicroRNA 21a-5p is induced by TGFβ1 and further promotes TGFβ1 signaling by targeting the inhibitory SMAD7 in a feed-forward regulatory loop, thereby promoting fibroblast proliferation and fibrosis (215). Finally, microRNA 29a-3p is a well-known suppressor of many prominent pro-fibrotic genes (216). The finding that miR-29 is downregulated in preclinical models therefore led to the investigation of miR-29 mimics as therapeutic agents to suppress multiple, possibly fibrosis-driving genes at once (217). Based on the successful identification of these important miRNAs, further candidates of the “hit” list were explored with regard to their function and putative targets.

Table 5: microRNAs anti-/correlated with lung function

<table>
<thead>
<tr>
<th>Rank</th>
<th>microRNA</th>
<th>r</th>
<th>AAV</th>
<th>Bleo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mmu-miR-676-3p</td>
<td>0.7854</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>mmu-miR-582-3p</td>
<td>0.7755</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>mmu-miR-151-3p</td>
<td>0.7549</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>mmu-miR-181a-5p</td>
<td>0.7419</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>mmu-miR-26b-5p</td>
<td>0.7418</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>mmu-miR-30c-2-3p</td>
<td>0.7293</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>mmu-miR-451a</td>
<td>0.7224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>mmu-miR-146a-5p</td>
<td>0.7212</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>mmu-miR-30d-5p</td>
<td>0.7165</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>mmu-miR-1486-3p</td>
<td>0.7150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>mmu-miR-3107-5p</td>
<td>0.7150</td>
<td></td>
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<tr>
<td>12</td>
<td>mmu-miR-3107-3p</td>
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<tr>
<td>13</td>
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<tr>
<td>14</td>
<td>mmu-miR-26a-5p</td>
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<tr>
<td>15</td>
<td>mmu-miR-200a-5p</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>mmu-miR-181b-5p</td>
<td>0.6889</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>mmu-miR-29a-3p</td>
<td>0.6861</td>
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<tr>
<td>18</td>
<td>mmu-miR-31-5p</td>
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<td>19</td>
<td>mmu-miR-144-5p</td>
<td>0.6771</td>
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<td>20</td>
<td>mmu-miR-10a-5p</td>
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</table>

Table 5 shows miRNAs commonly deregulated during the fibrotic phase of the Bleomycin- and AAV-TGFβ1-induced models of fibrosis, whose expression profiles were strongly correlated (left table) or anti-correlated (right table) with the decrease in lung function observed over time (compare Figure 20). The miRNAs were sorted by their mean Pearson correlation coefficient r and the expression profiles depict individually scaled log2fc changes over all experimental time points (3, 7, 14, 21, 28 days).

For the prediction of putative miRNA targets, a list of miRNAs was applied to the multiMiR database search (218), which in turn queries data from eleven other public miRNA-target prediction platforms, including both solely prediction-based and experimentally validated targets.
Results

We defined a putative target as a “hit”, when it was predicted by at least two out of eleven databases. The list of putative targets was then limited to such mRNAs that were differentially expressed in our experiments (at least at one time point in one of our models) and further, whose expression profile over time was anti-correlated to that of its putative miRNA regulator (r <-0.65). Following this strategy, a list of putative, anti-correlated mRNA targets was retrieved for each of the deregulated miRNAs.

Considering common deregulation in both models, anti-correlation of miRNA and mRNA, anti-/correlation with lung function, the lists of putative targets and published literature, several possibly fibrosis-relevant miRNAs were identified, including miR-335-3p, miR-214-5p, miR-144-5p and miR-200. In addition, two of the most interesting miRNAs identified were miR-676-3p and miR-181a-5p, which both were strongly correlated with the decrease in lung function. For miR-181a-5p, 229 potential targets in our models were identified, many of which were associated with ECM organization, including elastin, several collagen family members, PAI-1, LOX, TGFβ receptor type I and integrin β8 (Figure 28). In contrast, for miR-676-3p relatively specific targeting of collagen 11 α1 and hyaluron synthase 2 (HAS2) was suggested. Another miRNA, miR-192-5p caught our attention, because it was predicted as a potential regulator of LOXL2 along with collagens 3 α1 and 5 α1 as well as tenascin C (TNC). Notably, these examples also illustrate, how different miRNAs might act in concert to suppress the same target, as suggested for miR-181a-5p and miR-192-5p with regard to the collagen family members 3 α1 and 5 α1 (Figure 28). Although these results are largely prediction-based, they nevertheless represent a promising starting point for further investigation of the role of miR-181a-5p, miR-192-5p and miR-676-3p and their putative targets, including LOXL2 and HAS2, in pulmonary fibrosis. Given that all three miRNAs were downregulated in our experiments, our results suggest that overexpression of these miRNAs might have the potential to attenuate pulmonary fibrosis.

Taken together, the whole transcriptome analysis of two models of pulmonary fibrosis based on different modes of disease induction enabled in-depth insight into potential regulatory mechanisms relevant for disease onset, maintenance and progression. The data derived from these experiments, including temporal mRNA and miRNA expression profiles, predicted miRNA-mRNA interactions and lung function measurements along with publically available protein-protein interactions and protein properties such as cellular localization, are currently being integrated into a complex, multi-dimensional network. This network will allow for simultaneous multifactorial analyses, which will likely lead to the identification of novel regulatory pathways and new drug target candidates.
Results

Following the criteria described in Table 2, all genes commonly deregulated during the fibrotic phase upon both Bleomycin and AAV-TGFβ1 treatment were analyzed for enriched “GO Biological processes” using EnrichR. All genes associated with the process “ECM organization” are depicted in this word cloud and the font size corresponds to their mean log2-fold change during the fibrotic phase across Bleomycin and AAV-TGFβ1-treated animals, which was calculated as described in the legend of Table 2. For miR-181a-5p, miR-192-5p and miR-676-3p, putative mRNA targets were predicted using multiMiR and limited to targets, whose expression profile over time was anti-correlated to the miRNAs (see text for detailed procedure). Putative targets among the ECM organization-associated genes are presented and were color-coded according to their putative miRNA regulator.

In summary, the multilayered phenotypic and transcriptomics approach provided important insight into the molecular changes likely involved in inducing and maintaining fibrosis and further demonstrates the value of integrating several models for the identification of critical disease pathways.
2.2.4 Pharmacological intervention

As demonstrated above, the AAV-TGFβ1 fibrosis model can be used to elucidate critical mediators and pathways involved in disease biology and might further serve as a source for the identification of novel potential drug targets. Equally importantly, such models can be used for the preclinical pharmacological profiling of drug candidates or for in vivo target validation, for instance by antagonizing a target of interest by pharmacological intervention using a tool compound and studying subsequent effects on the disease phenotype. Therefore, an important question was, whether it is feasible to pharmacologically intervene in the AAV-TGFβ1 model.

As an initial proof-of-concept study, the well-established, potent (IC$_{50}$ = 14.3 nM) and largely selective (~4-fold over ALK4, >1000-fold over ALK2, ALK3, ALK6) ALK5 (TGFβ receptor type I) inhibitor SB-525334 (219) was tested in the TGFβ1 model. For this purpose, mice received AAV-TGFβ1 by i.t. administration. From day 7, the mice were treated with 30 mg/kg SB-525334 either once or twice daily by oral administration. After a total of 21 days, mice were killed and the effect of ALK5 inhibition was assessed relative to AAV-TGFβ1 control mice that had only received vehicle.

When monitoring body weight over time, a strong decrease in weight was observed in vehicle-treated mice from day 8, whereas mice that had received the ALK5 inhibitor, gained in weight in a similar way as did the animals that had not received AAV-TGFβ1 (Figure 29a). Interestingly, a trend towards a slightly higher bodyweight of animals that had received twice daily as opposed to once daily treatment was also observed.

![Figure 29: Body weight and TGFβ1 BAL levels of AAV-TGFβ1 mice treated with SB-525334](image)

Mice either received PBS or 2.5x10$^{11}$ vg AAV-TGFβ1 by i.t. administration on day 0 and were treated with 30 mg/kg of SB-525334 or vehicle from day 7 by oral (gavage) application, either once (o.d.) or twice (b.i.d.) daily. (a) Body weight was tracked every day for the full duration of the 3 week experiment. (b) TGFβ1 protein levels were measured in BAL samples using ELISA. n= 3 (PBS), 4 (vehicle o.d.), 5 (vehicle b.i.d.) and 8 (ALK5i) animals per group. Mean +/- SEM. */# p<0.05, **/##p<0.01, ###/####p<0.001. In (a) statistical significance for o.d. treatment relative to o.d. vehicle is indicated by hashmarks, whereas asterisks indicate statistical significance for the b.i.d. group relative to its respective vehicle control group.
To confirm that the observed differences in body weight were not due to differences in TGFβ1 expression following AAV application, TGFβ1 protein levels were measured in BAL samples of the animals. While the results showed that compound-treated animals showed at least equal levels of TGFβ1 expression as compared to vehicle-treated mice, a surprising and highly significant increase in total TGFβ1 levels was observed specifically in animals that had received the ALK5 inhibitor twice daily (Figure 29b). These results might suggest that ALK5 blockage by SB-525334 prevents the normally occurring, endocytic internalization of the TGFβ1-TGFβ-receptor type I/II complex (220), thereby causing an accumulation of freely available, unbound TGFβ1 protein.

Analysis of immune cells showed that twice daily compound treatment was efficacious in suppressing the TGFβ1-induced influx of total immune cells and more specifically, monocytes and lymphocytes (Figure 30). Notably, despite the clear improvement of body weight by once daily administration, at best a trend towards less immune cell influx was observed in these animals.

**Figure 30: Differential immune cell counts in AAV-TGFβ1 mice upon treatment with SB-525334**

AAV-TGFβ1-treated mice were treated with the ALK5 inhibitor SB-525334 as described in detail above (see Figure 29) and differential immune cell counts were determined in the BAL on day 21 post AAV application (i.e. 14 days post compound treatment). WBC= white blood cells, Mono= monocytes, Neutro= neutrophils, Lympho= lymphocytes. n= 3 (PBS), 4 (vehicle o.d.), 5 (vehicle b.i.d.) and 8 (ALK5i) animals per group. Mean +/- SEM. */#/ p<0.05, **/##p<0.01, ***/###p<0.001. Hashmarks and asterisks indicate statistical significance relative to PBS-treated animals and respective vehicle control groups, respectively.
Similar to our previous observations, mice receiving AAV-TGFβ1 and vehicle treatment showed a strong gain in lung weight, however, this increase was strongly attenuated by ALK5 inhibition with twice daily treatment being more efficient as once daily administration (Figure 31a). Compound treatment also significantly decreased fibrosis, as evident from both, histological assessment (Ashcroft scoring, Figure 31b) and – very drastically – from micro-CT analysis, where an almost full protection was observed (Figure 31c). In line with these findings, also a significant improvement (+25.3 %) in lung function was observed in animals that had received twice daily treatment (Figure 31d). While at first sight this improvement seems small, it is noteworthy that at the start of treatment (i.e. seven days post AAV-TGFβ1 application), already a 20 % decrease was observed in previous experiments (Figure 20c). Although limited by the fact that two independent experiments are compared, these findings might still suggest that the 25.3 % increase in lung function could actually correspond to 59.7 % protection, when calculated relative to day seven, where compound treatment was started.

Figure 31: Lung weight, fibrosis manifestation and lung function upon treatment with SB-525334
AAV-TGFβ1-treated mice were treated with the ALK5 inhibitor SB-525334 as described in detail above (see Figure 29) and analyses were conducted 21 days following AAV administration. (a) Wet lung weight. (b) Histological examination (Ashcroft score). (c) micro-CT analysis of fibrotic lung volume. (d) Lung function (compliance) measurements. n= 3 (PBS), 4 (vehicle o.d.), 5 (vehicle b.i.d.) and 8 (ALK5i) animals per group. Mean +/- SEM. */# p<0.05, **/##p<0.01, ###/###p<0.001. Hashmarks and asterisks indicate statistical significance relative to PBS treatment and the respective vehicle control groups, respectively.
Results

Taken together, using the ALKS inhibitory compound SB-525334, most fibrosis-associated effects triggered by AAV-mediated TGFβ1 expression could be strongly attenuated, resulting in a much improved overall health status of the animals (as evident from a close-to-normal gain in body weight), less fibrosis and improved lung function. Therefore, the results clearly demonstrate principal feasibility of pharmacological intervention in the AAV-TGFβ1 model of pulmonary fibrosis.

2.3 Riboswitches as novel tools to regulate AAV transgene expression

As comprehensively demonstrated above, AAV vectors resemble novel, favorable vectors for the characterization of transgene function in vivo and setup of preclinical disease models. One prerequisite for the broader application of such vectors is the easy manufacturing of high-titer vector preparations that allow carrying out studies in a sufficient number of animals and ideally, in several independent experiments using the same characterized AAV batch. Transient transfection of HEK-293 cells using several co-provided plasmids harboring Adenoviral helper genes, AAV capsid and replication genes as well as an ITR-flanked expression cassette containing the transgene of interest, is the most frequently used method for AAV production. However, due to the fact that the majority of studies carried out to date make use of constitutively, often ubiquitously active promoters (for instance the CMV promoter), efficient vector production can be hampered by the expression of the transgene of interest during vector production in HEK-293 cells. Transgene expression not only burdens the cells’ translational capacity for the production of an unnecessary gene product, but might also induce pro-apoptotic, cell cycle-modulating or other unknown effects that interfere with producer cell performance and therefore reduce viral vector yield. In fact, the AAV6.2-CMV-TGFβ1 vector used in the studies described above could only be produced at sufficient titer, when large batches of >100 cell culture dishes were produced, likely as a result of TGFβ1 protein expression during AAV production.

Speculating that suppressing transgene expression during AAV production would increase viral vector yield, we were aiming to develop a vector system allowing transgene regulation in HEK-293 cells. To enable broad applicability and minimal immunogenicity, we envisioned a system that works independently of additionally expressed transcription factors, allows rapid adaptability to different transgenes and promoters and is relatively small – an important property in the context of AAV vectors, due to their limited packaging capacity of ~4.7 kb (221). Therefore, we explored artificial riboswitches for the conditional regulation of transgene expression during AAV production (see scheme in Figure 32a).
2.3.1 Riboswitch-mediated control of transgene expression in HEK-293 cells

To identify a riboswitch construct that enables efficient regulation of transgene expression under the conditions used for AAV production, pAAV plasmids harboring an eGFP gene under the control of a CMV-promoter and the guanine-responsive GuaM8HDV riboswitch in either the 3’-UTR, 5’-UTR or at both positions were generated (Figure 32a). GuaM8HDV is a recently engineered, guanine-responsive riboswitch based on a guanine aptamer derived from the 5’-UTR of the Bacillus subtilis xpt-pbuX operon (222), which was fused to the hepatitis delta virus ribozyme (223) and that has been previously shown to enable reporter gene control in HEK-293 cells (224).

HEK-293 cells were transiently transfected with either 5’, 3’ or 5’3’-GuaM8HDV-eGFP constructs and 5 h later, the medium was replaced with fresh medium containing increasing guanine concentrations of up to 400 µM. 24 h after transfection, GFP fluorescence was analyzed by microscopy and flow cytometry. While transfection with the riboswitch-free control construct led to stable GFP expression, which was not affected by guanine addition, both the 5’- and 5’3’-GuaM8HDV constructs showed strongly decreased GFP expression even in absence of guanine, which further decreased with increasing guanine concentration (Figure 32b). In contrast, the 3’-GuaM8HDV construct retained approximately 90% of GFP expression when no guanine was present, which could be dose-dependently suppressed by guanine addition, approaching a minimum of ~25% residual GFP expression at 200 µM guanine (Figure 32b). Microscopic analysis further underscored these results (Figure 32c). Moreover, microscopy revealed a slight decrease in cell numbers at 400 µM guanine that, however, was not associated with increased LDH release (Figure 33a), suggesting that guanine concentrations >400 µM might exert slight anti-proliferative but no prominent cytotoxic effects. For AAV production, transfected HEK-293 cells are usually kept in culture for 72 h. Importantly, using the 3’-GuaM8HDV construct and a single addition of guanine, GFP expression remained stably suppressed over the required time period of 72 h (Figure 32d) which again was also validated by microscopy (Figure 32e). When the cells were further incubated for an additional 4 days, a slight continuous increase in residual GFP expression was observed; however, this increase could be fully blocked by a second addition of guanine 72 h after transfection (Figure 33b). Taken together, the pAAV construct harboring GuaM8HDV in its 3’-UTR effectively attenuates transgene expression in a guanine dose-dependent manner.
**Results**

Figure 32: 3’-GuaM8HDV enables suppression of GFP expression in HEK-293 AAV producer cells

(a) Scheme of AAV-GuaM8HDV construct design and riboswitch-mediated transgene suppression. The riboswitch was inserted either upstream (5’) or downstream (3’) of the transgene, whose expression is driven by a CMV promoter. The transgene cassette is flanked by AAV2 ITR sequences, which define the DNA section that is packaged into the AAV particle. While transgene expression in absence of the ligand (guanine) might lead to toxic effects that can decrease AAV vector yield (upper panel), guanine addition triggers self-cleavage of the riboswitch, which attenuates transgene expression, thereby increasing AAV vector yield (lower panel).

(b) Normalized GFP expression measured by flow cytometry and (c) fluorescence microscopic analysis of HEK-293 cells 24 h after transfection with either 3’-GuaM8HDV-harboring pAAV-GFP or a riboswitch-free pAAV-GFP control construct and addition of increasing concentrations of guanine. n= 4, mean +/- SD. Scale bar= 400 µm. (d) Normalized GFP expression measured by flow cytometry and (e) fluorescence microscopic analysis of HEK-293 cells 24, 48 and 72 h after transfection with the 3’-GuaM8HDV-harboring pAAV-GFP construct and addition of increasing concentrations of guanine. ctrl= 0 µM guanine. n= 3, mean +/- SD. *p<0.05, **p<0.01. Scale bar= 400 µm.
Results

Figure 33: Assessment of toxicity and stability associated with guanine-mediated expression control

HEK-293 cells were either transfected with a GFP-expressing control construct or a 3'-GuaM8HDV-harboring GFP-expressing plasmid. (a) 5 h after transfection, guanine was added to the medium at increasing concentrations. 24 h after transfection, cytotoxicity was assessed by measuring LDH-release in the cell supernatant. Triton-lysed, untransfected cells served as a positive control (pos) and were set to 100%. n=4, mean +/- SD. (b) 5 h after transfection, guanine was added to the medium at 200 µM and optionally again at 72 h after transfection (black bars). Direct GFP fluorescence was measured at each time point and is expressed relative to the GFP control plasmid, which was set 100%. n=6, mean +/- SD. **p<0.01.

After having identified the 3’-GuaM8HDV construct as a suitable vector backbone, we next asked whether riboswitch-mediated transgene regulation would allow suppressing the functional effects of genes known to impair host cell integrity upon (over-) expression and thus might negatively influence AAV vector production. To this end, the GFP gene was replaced by the genes encoding BAX, TNFα, TGFβ1, LOXL2 and PAI-1, respectively. While BAX and TNFα are well-known inducers of apoptosis (225)(226), TGFβ1 is involved in many processes such as cell growth, cell-cycle regulation and cell differentiation (see introduction). In contrast, LOXL2 has not been reported in the context of cytotoxicity so far, however, attempts to produce LOXL2-expressing AAV vectors for protein characterization studies failed due to very low viral vector yield, indicating producer cell impairment of yet unknown cause. Finally, as mentioned above, PAI-1 is a well-known downstream target of TGFβ1 (203) and an important regulator in the context of coagulation. Moreover, we observed HEK-293 cell detachment upon overexpression of PAI-1 (likely due to competitive displacement of cell-adhesion molecules by PAI-1’s vitronectin binding domain (227)), which is why we speculated that PAI-1 might negatively influence AAV production as well. The expression of these genes and whether associated functional effects can be controlled by the riboswitch was studied in transiently transfected HEK-293 cells (Figure 34).
Figure 34: 3’-GuaM8HDV attenuates transgene-mediated impairment of producer cell integrity

(a) Western Blot analysis of BAX (23 kDa) expression in HEK-293 cell lysates 16 h after transfection with either the 3’-GuaM8HDV-harboring pAAV-BAX construct or a riboswitch-free pAAV-BAX control (ctrl) construct and addition of indicated amounts of guanine. Loading control: anti-Vinculin (116 kDa) staining. (b) ELISA measurement of TNFα protein levels in the supernatant (sup.) of HEK-293 cells, 24 h after transfection with either the 3’-GuaM8HDV-harboring pAAV-TNFα construct, a riboswitch-free pAAV-TNFα ctrl or mock (mo) and addition of guanine. n= 6, mean +/- SD. (c, d) Cytotoxicity analysis by lactate dehydrogenase (LDH) detection in the sup. of HEK-293 cells 24 h after transfection with either (c) the 3’-GuaM8HDV-harboring pAAV-BAX construct or a riboswitch-free pAAV-BAX ctrl or (d) the 3’-GuaM8HDV-harboring pAAV-TNFα construct or a riboswitch-free pAAV-TNFα ctrl and addition of guanine. Triton-lysed cells served as a positive ctrl and were set 100 %. n= 5, mean +/- SD. (e) ELISA detection of TGFβ1 protein levels in the sup. and (f) qPCR-based measurement of PAI-1 gene expression (as a downstream marker of TGFβ1 signaling) in RNA samples of HEK-293 cells, 24 h after transfection with either the 3’-GuaM8HDV-harboring pAAV-TGFβ1 construct or a riboswitch-free pAAV-TGFβ1 ctrl and addition of guanine. n= 4, mean +/- SD. (g) Western Blot analysis of LOXL2 (87 kDa) expression in the sup. of HEK-293 cells 48 h after transfection with either the 3’-GuaM8HDV-harboring pAAV-LOXL2 construct or a riboswitch-free pAAV-LOXL2 ctrl and addition of guanine. (h) ELISA measurement of PAI-1 protein levels in the sup. and (i) microscopic analysis of the cells (20x), 72 h after transfection with either the 3’-GuaM8HDV-harboring pAAV-PAI-1 construct or a riboswitch-free pAAV-PAI-1 ctrl and addition of indicated amounts of guanine. n= 4, mean +/- SD. *p<0.05, **p<0.01, ***p<0.001 relative to ctrl 0 µM or as indicated. ###p<0.001 relative to untreated cells.
As expected, guanine addition to transfected cells dose-dependently downregulated transgene expression in all cases tested (Figure 34a, b, e, g, h). Importantly, this decrease translated into an attenuation of cytotoxic and cell performance-impairing effects: Specifically, while TNFα and BAX expression induced cytotoxicity, as measured by an increased release of LDH, GuaM8HDV-mediated suppression (Figure 34a, b) decreased cytotoxic effects by about 40% in the case of BAX (Figure 34c) and to baseline levels in the case of TNFα (Figure 34d). Moreover, riboswitch activation successfully reduced TGFβ1 protein levels (Figure 34e) and associated downstream signaling as assessed by gene expression measurement of the surrogate marker (203) gene PAI-1 (Figure 34f). Furthermore, GuaM8HDV activation successfully reduced LOXL2 protein expression in a dose-dependent manner (Figure 34g). Finally, riboswitch-mediated attenuation of PAI-1 expression (Figure 34h) fully blocked the PAI-1-mediated detachment of HEK-293 cells (Figure 34i). Thus, GuaM8HDV-mediated regulation of transgene expression effectively suppressed unwanted effects exerted by the selected genes.

2.3.2 Riboswitch-mediated increase in AAV vector yield

During the production of AAV vectors in HEK-293 cells, transgenes are expressed as an unnecessary byproduct, which might interfere with producer cell performance. We thus asked, whether riboswitch-mediated attenuation of transgene expression during viral vector production would increase the yield of respective AAV vectors. In a proof-of-concept approach, AAV vectors harboring the genes encoding TNFα, BAX, TGFβ1, LOXL2 and PAI-1 under the control of a CMV promoter with and without the GuaM8HDV riboswitch placed in the 3'-UTR were produced. To examine potential differences between AAV variants, each of these genes were packaged in AAV6.2, AAV8 and AAV9 – three of the most efficient and commonly used vectors for gene transfer to the lung, liver and brain (81)(91), respectively.

For AAV production, HEK-293 cells were transfected with plasmids encoding the AAV rep/cap and adenoviral helper genes along with a construct carrying the gene of interest flanked by AAV ITRs. 5 h after transfection, the medium was replaced with fresh medium, optionally supplemented with 100 µM or 200 µM guanine. The results show that in all cases tested except from PAI-1, transgene suppression led to an increase in AAV yield (Figure 35). Specifically, using the riboswitch-free control construct, AAV-BAX yields were very low (1x10⁸ vg) due to strong cytotoxicity (Figure 34c), whereas upon guanine addition a dose-dependent, up to 23-fold increase (AAV6.2) was achieved. Interestingly, TNFα seemed to be less potent regarding apoptosis-induction (Figure 34d), which translated into initially higher AAV yields using the control construct (2-4x10⁹ vg). Nevertheless, using GuaM8HDV, yields could be further increased by up to 2.3-fold (AAV8).
Results

Figure 35: 3’-GuaM8HDV-mediated suppression of toxic transgene expression increases AAV yields

Benzonase-resistant AAV vector genomes (VG) were quantified by qPCR in HEK-293 cell lysate, 72 h after transfection with either the 3’-GuaM8HDV pAAV-BAX, -TNFα, -TGFβ1, -LOXL2 or -PAI-1 construct or respective riboswitch-free control constructs (and further plasmids necessary for the production of AAV6.2, AAV8 or AAV9 – see Materials & Methods section for details) in presence of indicated concentrations of guanine, which were added during the medium exchange step about 5 h after transfection. n= 3 biological replicates, mean +/- SD. *p<0.05, **p<0.01, ***p<0.001.
Moreover, while both AAV-TGFβ1 and -LOXL2 yields were in the range of 1-5x10^9 vg using conventional constructs, a 3- to 5-fold increase of AAV-yields was obtained by riboswitch activation. Contrary to our expectations, PAI-1-mediated cell detachment did not impair AAV yields in the first place, which is why no further improvement in AAV titers was observed using the riboswitch construct. Notably, while the absolute yields of AAV6.2, -8 and -9 were partly different, the riboswitch effects were independent of the AAV capsid variant used.

We finally directly compared the yields of AAV-BAX-, -TNFα-, -TGFβ1- and -LOXL2-riboswitch vectors to those obtained using conventional or riboswitch-carrying AAV-GFP constructs. In the first place, our data show that neither guanine nor the presence of GuaM8HDV per se negatively influence AAV vector yield. In fact, it rather seems that also in the case of GFP, riboswitch-activation might be beneficial, as a 2-fold increase in AAV-GFP yields was observed (Figure 36). Furthermore, by using the riboswitch system, the yields of AAV-TNFα, -TGFβ1 and -LOXL2 vectors could be increased to or beyond the levels of conventional AAV-GFP vectors (Figure 36). Despite a strong increase, AAV-BAX levels only reached 7% of the titer of the AAV-GFP vector. Taken together, riboswitch-mediated suppression of toxic transgene expression increased AAV yields, while the effect size seemed to be dependent on the severity of initial toxicity.

Figure 36: The 3’-GuaM8HDV riboswitch system enables high-titer vector production
AAV6.2 vectors carrying various transgene constructs were produced as described in Figure 35 in the presence or absence of 150 µM guanine (as indicated). 72 h after transfection, benzonase-resistant AAV vector genomes (VG) were quantified by qPCR in HEK-293 cell lysate. The percentage of vector titers relative to the riboswitch-free GFP control construct is depicted on each bar. The AAV-GFP yield range is indicated by the striped background. 3’-Gua = 3’GuaM8HDV. n= 6, mean +/- SD. *p<0.05, **p<0.01, ***p<0.001, relative to GFP without guanine.
Since the observed increase in viral vector yield is only valuable, if riboswitch-containing AAV vectors preserve functionality, i.e. their ability to express the transgene in vivo, we finally explored transgene expression and functional downstream effects by a 3′-GuaM8HDV-harboring AAV vector in mice. For this purpose, we made use of the AAV-TGFβ1 model of pulmonary fibrosis, described in detail in the previous chapter. To this end, the 3′GuaM8HDV-harboring AAV6.2-TGFβ1 vector was first produced at larger lab-scale. Notably, the results obtained in 6-well microplates (Figure 35) could be successfully scaled up to larger lab scale (i.e. 40 x 15 cm dishes), yielding 3.9-fold higher AAV titers (2.2x10^{13} vg) with the riboswitch in presence of 150 µM guanine as compared to previous productions (5.6x10^{12} vg) with the control construct. For the in vivo assessment of TGFβ1 expression, AAV6.2-TGFβ1 vectors with or without the GuaM8HDV riboswitch in their 3′-UTR were then applied to the lung of mice by intratracheal administration and fibrosis manifestation was assessed 3 weeks after application. Anticipating that the riboswitch-harboring construct (due to the minimally constitutive riboswitch activity in vitro (see Figure 32)) might show slightly lower expression than the control construct, both, an equal and a 1.5-fold higher dose of the 3′GuaM8HDV vector compared to the TGFβ1 control vector were applied.

The results demonstrate that TGFβ1 protein levels in lung lavage samples were slightly higher in mice that received the conventional AAV-TGFβ1 vector (ctrl) as compared to the same dose of the riboswitch-harboring vector (Figure 37a). However, this difference could be fully compensated by applying a 1.5-fold higher dose of the 3′-GuaM8HDV vector. The differences in TGFβ1 levels also translated into differences in immune cell influx, where both the control and high-dose riboswitch group showed higher levels than the low dose switch vector (Figure 37b). Directly evident of increased extracellular matrix (ECM) deposition, which is a hallmark of tissue remodeling in pulmonary fibrosis, an increase in lung weight was observed in all TGFβ1-overexpressing animals (Figure 37c). Again, the increase triggered by the TGFβ1 control vector (1.55-fold) was significantly higher than that induced by the switch-harboring construct (1.24-fold). However, by using a 1.5-fold higher dose of the switch vector, these differences could be completely abolished. To assess the structural changes associated with fibrotic tissue remodeling, lung tissue slices were analyzed by Masson-trichrome staining (Figure 37d) and subsequent pathological scoring (Ashcroft score) of disease severity (Figure 37e), which further validated the observed differences and similarities among the treatment groups.
Results

Figure 37: 3’-GuaM8HDV-harboring AAV vectors are functional in vivo
Mice received a single application of either $2.7 \times 10^{11}$ vg of a riboswitch-free AAV6.2-CMV-TGFβ1 control vector, the same dose or a 1.5-fold higher dose ($4.0 \times 10^{11}$ vg) of the 3’-GuaM8HDV-containing AAV6.2-CMV-TGFβ1 vector or PBS via i.t. administration. Analyses were conducted 21 days after application. (a) ELISA measurement of TGFβ1 protein levels in bronchoalveolar lavage (BAL) samples. (b) Total immune cell counts measured in BAL samples. (c) Wet lung weight. (d) Masson-trichrome staining of FFPE lung tissue sections and (e) corresponding Ashcroft score. (f) Lung function analysis. n= 5 animals per group, mean +/- SEM. *p<0.05, **p<0.01, relative to PBS-treatment or as indicated. Scale bar= 200 µm. switch= 3’GuaM8HDV.

Finally, as the ultimate consequence of fibrotic lung tissue scarring, impairment of lung function was observed in all AAV-TGFβ1-treated animals (Figure 37f). While the decrease in lung compliance was higher in TGFβ1 control vector treated animals (-38.0 %) than in animals receiving an equal dose of riboswitch-TGFβ1 vector (-26.4 %), no statistically significant differences were observed when a 1.5-fold higher dose of riboswitch-vector (-32 %) was applied. Taken together, the 3’-GuaM8HDV-harboring AAV vector successfully induced TGFβ1 expression in the lung of mice, which triggered expected pathological changes at similar efficiency as the riboswitch-free control vector. Thus, the riboswitch approach described herein represents an innovative approach for the efficient production of AAV vectors that harbor producer cell integrity-impairing transgenes.
2.3.3 Riboswitch-mediated control of AAV-mediated transgene expression

Besides its use as a plasmid-based regulator, for instance for the use in AAV production, riboswitches also represent promising tools for the control of transgenes delivered by viral vectors, both, as a research tool and for potential therapeutic applications, i.e. viral gene therapy. In gene therapeutic approaches, constitutive transgene expression is sufficient for various applications and also desirable in many cases, e.g. for the treatment of monogenetic disorders, where a steady therapeutic level of transgene expression should be achieved. However, for other therapeutic strategies such as oncolytic or anti-inflammatory approaches, switchable gene expression is highly desirable. Here, regulatable expression could serve as a safety switch to turn off transgene expression in cases of severe side effects, for example in oncolytic virotherapy. In turn, the ability to transiently switch on transgene expression only for the time of therapeutic treatment could be desirable in cases, where constitutive expression would be likely to impair immunological homeostasis, for example in anti-inflammatory and immunosuppressive gene therapy. Therefore, an important question was, whether the riboswitch also allows controlling transgene expression upon AAV-mediated transfer.

To explore this question, AAV6.2 vectors harboring either a control eGFP transgene cassette or a eGFP-3’-GuaM8HDV cassette were produced in HEK-293 cells. After lysing the cells by three freeze/thaw cycles and pelleting cell debris by centrifugation (20,000 xg), the AAV-containing supernatant was added to HEK-293 cells at increasing amounts. After seven hours, the medium was replaced, optionally supplemented with 150 µM guanine. After three days, AAV-mediated eGFP expression was assessed by measuring GFP intensity using a microplate reader (Figure 38).

![Figure 38](image.png)

**Figure 38: 3’-GuaM8HDV enables regulation of AAV-mediated transgene expression in vitro**

HEK-293 cells were transduced with either AAV-eGFP control or 3’-GuaM8HDV-harboring AAV-eGFP vectors at increasing amounts (µL of AAV-containing HEK-293 cell lysate). 7 hours after AAV addition, the medium was replaced with fresh medium, optionally supplemented with 150 µM guanine. 72 h after transduction, GFP expression was assessed by measuring the mean fluorescence intensity (MFI) using a plate fluorescence reader. n=2, mean +/- SD. **p<0.01, ***p<0.001.
The results show that AAV-mediated GFP expression in HEK-293 cells was unaffected by guanine addition, when riboswitch-free control vectors were used (Figure 38, left graph). In contrast, GFP fluorescence could be suppressed by at least 4-fold using AAV-eGFP-3’GuaM8HDV vectors in presence of guanine (Figure 38, right graph), thereby demonstrating the possibility to control AAV-mediated transgene expression in vitro.

Following the positive results using AAV-containing HEK-293 lysate, we aimed to confirm these results with purified vectors and known AAV titers. Moreover, in addition to studying the effect of increasing guanine concentrations, the possibility of dynamic regulation of expression should be explored. To this end, HEK-293 cells were transduced with purified AAV-eGFP-3’GuaM8HDV vector in presence or absence of increasing doses of guanine. The effect of guanine treatment was assessed over a period of three days after AAV application by measuring GFP expression using a fluorescence plate reader. The results show that upon transduction with AAV at a high multiplicity of infection (100,000), GFP expression could be guanine dose-dependently suppressed by factor 4, corresponding to a residual GFP fluorescence of 25 % three days after vector transduction (Figure 39, left graph).

In order to explore whether transgene expression can be dynamically switched off, guanine (150 µM) was then added to cells that were previously grown in absence of guanine, whereas the other half of these cells were kept guanine-free as a control. In turn, to study whether GFP expression can be restored in cells, where expression was previously suppressed by the addition of guanine, half of the cells previously grown in presence of 200 µM guanine were henceforth grown in the absence of guanine, or further cultured in medium containing 150 µM guanine. After three additional days of growth under these conditions, GFP fluorescence was measured again.

As expected, cells that were continuously grown in absence of guanine still showed stable GFP fluorescence. However, strikingly, the cells treated with guanine 72 h after transduction now demonstrated strongly reduced GFP expression at about 40 % as compared to control cells (Figure 39, upper right panel). In turn, cells that were continuously grown in presence of guanine kept showing attenuated eGFP expression (~20 %). In stark contrast, when guanine was removed three days after transduction, eGFP expression was restored to 75 % as compared to control cells that were grown guanine-free (Figure 39, lower right panel). Thus, these results not only confirm that AAV-mediated transgene expression can be controlled by the GuaM8HDV riboswitch in vitro, but also demonstrate the principle feasibility of dynamic expression regulation.
Results

Figure 39: 3'-GuaM8HDV allows for the dynamic regulation of AAV-mediated transgene expression

HEK-293 cells were transduced with a 3'-GuaM8HDV-harboring AAV-eGFP vector at a MOI of 100,000 in absence or presence of increasing amounts of guanine, as indicated. GFP expression was followed over three days and determined by measuring the mean fluorescence intensity (MFI) using a fluorescence plate reader. 72 h after transduction, the medium of half of the cells initially grown in absence of guanine (white bar) was replaced with fresh, guanine-free medium, whereas the medium of the other half of cells was supplemented with 150 µM guanine. Similarly, the medium of half of the cells initially grown in presence of 200 µM guanine (black bar) was replaced with fresh, guanine-supplemented (150 µM) medium, whereas the other half of cells received fresh guanine-free medium. Additional 72 h later (144 h), GFP expression was determined again. GFP fluorescence at 144 h is depicted in the two boxes in the right part of the figure. n= 12 (0 and 200 µM), n= 4 (50 and 100 µM). Mean +/- SD. **p<0.05, ***p<0.001.

Taken together, the results demonstrate that artificial riboswitches can be useful tools for various aspects in the context of viral vectors, such as optimization of vector production by suppressing unwanted transgene effects and control of transgene expression upon AAV-mediated delivery. Therefore, riboswitch engineering holds the potential for the development of inducible gene regulation systems for the use in viral vector gene therapy.
Results
3 Discussion

Modeling pulmonary fibrosis by AAV-mediated TGFβ1 expression – a proof of concept study for AAV-based disease modeling and riboswitch-controlled vector production
3.1 AAV as a tool for disease modeling and drug discovery research

Genetically modified animals, including transgenic and knockout mice, have been classically used to study gene function and induce disease phenotypes in preclinical models. As an alternative approach, viral vectors have been applied successfully to modulate gene expression in vivo by delivering cDNA, siRNA or miRNA to various organs and tissues. Vectors based on Retrovirus, including Lentivirus (LV), Adenovirus (AdV) and Adeno-associated virus are among the most commonly used vectors for this purpose, due to their different properties, for instance genomic integration of retroviruses (which can be desired for stable transgene expression but comes with the risk of insertional mutagenesis) or the property to transduce non-dividing cells (LV, AdV, AAV).

For preclinical studies in the context of pulmonary diseases research, AdV vectors have been most commonly used (101), thereby exploiting their natural pulmonary tropism. However, adenoviral transgene expression is associated with virus-targeted immune responses, preventing stable transgene expression due to the T-cell-mediated clearance of transduced cells (106). Moreover, inflammation might alter relevant experimental readouts and blur the effects induced by the transgene of interest studied.

Recent research suggests that AAV vectors – in particular AAV6.2 – might be an efficient alternative; however, besides one reporter gene study that prove the high efficiency of AAV6.2 for lung gene transfer (81) and one further report, where this vector was used to overexpress FOXP3 to attenuate experimental asthma (100), no functional studies have been reported so far. Our results showing efficient pulmonary transduction after intratracheal administration not only confirm these previous observations, but also provide valuable information on the cell types transduced by AAV6.2, which were identified as bronchial epithelial and type II alveolar epithelial cells (AEC II). This finding offers the opportunity to specifically investigate gene function or alveolar damage in AEC II cells by transcriptional targeting, for example by using the surfactant protein C (SFTPC) promoter (228). The observation that widespread transduction across the whole lung could be achieved regardless of the broncho-concentric route of administration (intratracheal) further illustrates the value of the AAV approach. Nevertheless, an easy-to-implement change and possible optimization of vector administration would be the use of micro sprayer devices, which hold the potential to further reduce fluctuations in transgene expression spread by aerosolizing the AAV suspension (229).

As mentioned previously, Adenovirus vectors have been mostly used for functional studies in the lung of mice, which however, is associated with vector-induced inflammation, including T-cell responses, thereby preventing stable transgene expression (106)(230). Although in some cases it might be favorable to include a viral inflammatory component, for instance to exacerbate
inflammatory disease models or when virus infection is known to contribute to disease pathology, viral vectors with low immunogenicity are preferred for most mechanistic studies. In accordance with our assumption that AAV vectors, which are non-pathogenic, might be less immunogenic upon lung delivery, the direct comparison of early inflammatory events following administration of either second-generation Ad5- or AAV6.2-stuffer vectors demonstrated significant inflammation using the Adenovirus vector, but not AAV. In fact, while Ad5-stuffer vectors induced inflammatory cytokine release, including IL1β, IL6 and TNFα, and influx of neutrophilic granulocytes and lymphocytes, AAV6.2 did not induce any of these changes. The finding that TLR3, TLR9 and MYD88 were upregulated following Ad5 administration confirmed published results on the involvement of the TLR-MYD88 signaling pathway in Adenovirus-directed immune responses (104)(105)(230). Moreover, the identification of Ad5-induced CD4+ and CD8+ T-cells likely explains the transient nature of transgene expression in previous studies of AdV-mediated TGFβ1 and IL13 expression in mice, where transgene levels peaked at day 7-10 but returned to baseline at day 14 (102)(103). These studies also reported increased levels of neutrophils and macrophages as well as IL10 and IL12 using control vectors. In contrast, neither inflammation nor T-cell activation was observed using AAV6.2, which allowed for stable expression of TGFβ1 protein in the lung of mice, contributing to the continuous increase in fibrosis severity. In addition, despite the episomal nature of AAV vector genome persistence, stable GFP expression was still evident in the lung of mice 4 months after a single administration of vector, suggesting feasibility of long-term expression. In fact, in the absence of T-cell-mediated clearance of vector-transduced cells, stability of transgene expression should be solely dependent on target cell turnover. While the lifespan of different lung epithelial cell types is still unclear and probably dependent on various factors including spatial location (slower turnover in more distal regions) and damage by pollutants and irritants, current lineage tracing studies suggest that bronchial ciliated epithelial cells in mice housed in pathogen-free environment have a lifespan of 17 months (231).

In this regard, AAV-mediated manipulation of transgene expression in the lung might indeed be a viable alternative to classical, stably genetically altered mice. Contrary to breeding transgenic animals, the AAV approach allows to modulate gene expression in adult wild type animals, thereby preventing issues related to developmental effects of genetic alterations and associated requirements for inducible systems or problems arising from acquired passenger mutations. Moreover, the ever growing portfolio of natural and synthetic AAV capsid variants with different cell and tissue tropism together with transcriptional and miRNA-mediated targeting, will further allow establishing a toolbox for the manipulation of specific cell types of interest. For lung gene transfer, AAV4 could be an interesting candidate, as it was shown to specifically transduce lung and heart after systemic administration (91). In general, while comparative studies of different
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AAV serotypes so far almost solely focused on overall transduction efficiency, all candidates, including AAV4-7, 6.2 and 9 should be systematically re-evaluated to identify the exact cell types targeted by each variant and whether systemic application results in pulmonary endothelial or parenchymal transduction via transcytosis. Besides the fact that intravenous administration would greatly simplify the application of AAV vectors for pulmonary research, it might also prove useful to mimic mediators or insults that access the lung via the bloodstream and encounter pulmonary endothelial cells as their first “targets”, as for example the case in clinical Bleomycin-induced lung injury (191).

Importantly, our results demonstrate that AAV6.2-mediated pulmonary TGFβ1 overexpression leads to persistent and likely progressive fibrosis in mice, thereby establishing proof-of-concept for disease modeling by AAV-mediated gene transfer. The degree of fibrosis was AAV-TGFβ1 dose-dependent, demonstrating the ability to titrate and fine-tune biological effects. Moreover, using SB-525344, a potent inhibitor of the type I TGFβ receptor/ALK5, pharmacological inhibition of fibrosis development was feasible, thereby demonstrating the principle suitability of AAV-induced models for compound testing in drug discovery research. While efficacy of a TGFβ receptor inhibitor in a TGFβ1-driven model obviously was expectable, an important finding of this experiment was that it is possible to intervene with the high levels of the disease-driving protein that is continuously produced by the transduced cells. Moreover, the almost full blockage of fibrosis observed in most of our readouts, including clinically relevant computed tomography, defined an experimental window for the measurement of compound effects and also illustrated what kind of effect sizes can be expected from a very potent drug in this model. Furthermore, as discussed in the next paragraph, this experiment gave important insight into the timing and sensitivity of TGFβ1-induced effects in lung fibrosis. An important question that should be tackled in future experiments is, whether the model is also suitable for the pharmacological profiling of compounds that do not directly target the TGFβ pathway, such as Nintedanib (a VEGFR, FGFR and PDGFR triple-tyrosine kinase inhibitor) and Pirfenidone (target unknown) – the most efficacious drugs for human IPF therapy to date.

Besides disease modeling, the AAV approach also enables addressing a range of other scientific questions: By delivering cDNA encoding or si/miRNA targeting a gene of interest, pharmacological activation and antagonizing, respectively, can be simulated when no (tool) compounds are available. An alternative approach to knock down a gene of interest would be the AAV-mediated expression of Cre recombinase in “floxed” animals. Both overexpression and knockdown can also be used to validate the biological function of potential drug targets in vivo. By combining AAVs harboring different cDNAs, defined multigenetic models could be established and studying the
role of disease-modifying genes in combination with key disease drivers would be feasible. Moreover, by AAV-mediated reconstitution of knockout mice with (mutant versions of) the respective deleted protein, the function of proteins or protein domains could be studied. Similarly, by expressing human target gene cDNA in mice, testing of non-cross-reactive drug candidates in preclinical models should become feasible, provided that the human protein shows similar effects in mice. Finally, recent advances in gene editing technology, specifically TAL effector nucleases (TALEN), Zinc-finger nucleases (ZFN) and CRISPR/Cas9 technology in conjunction with AAV might offer the possibility to introduce genetic modifications in somatic tissue (232)(233)(234).

Taken together, AAV6.2-mediated lung gene transfer prove to be an efficient alternative to other genetic approaches, including adenoviral delivery, thus enabling improved disease modeling and target validation with minimal interference due to vector-induced immune responses.

### 3.2 AAV-TGFβ1- and Bleomycin-induced pulmonary fibrosis

As mentioned before, AAV6.2-TGFβ1 successfully induced fibrosis upon intratracheal administration in mice in a mostly dose-dependent fashion. Fibrosis remodeling was relatively homogenously distributed, as evident from histology and CT imaging, which showed scarring in all areas of the lung. Moreover, the composition of immune cells present three weeks after vector administration (macrophages, followed by neutrophils and lymphocytes) is similar to what is observed in human pulmonary fibrosis (134). When studying the temporal disease course upon AAV-TGFβ1 administration, in contrast to both, adenoviral TGFβ1 expression and Bleomycin-induced fibrosis, fibrosis development occurred in the absence of acute inflammation. Moreover, due to the use of codon-usage optimized transgenic TGFβ1, our NGS analysis was capable to distinguish between exogenous and endogenous protein, showing that no activation of endogenous TGFβ1 was detectable. This contradicts the assumption of Sime and colleagues, who speculated that endogenous TGFβ1 might drive disease progression in their Adenovirus-TGFβ1 model, where transgene expression was lost after 14 days, but disease persisted (102).

Additionally, because the Adenovirus control vector caused inflammation, it was unclear whether fibrosis in their model was promoted by the virally induced inflammation or solely dependent on TGFβ1. Our results now clearly demonstrate that TGFβ1 alone is capable of inducing the disease.

While at first sight, our results seem to support the notion, that fibrosis in general can occur in the absence of inflammation, a more detailed view on our data reveals that despite an early overexpression of TGFβ1 at day three, which further increases to a plateau at about 14 days, thereby exceeding TGFβ1 levels induced by Bleomycin by 15-fold, distinct phenotypic changes
were only evident from day 14 onwards. Notably, fibrotic changes as evident from, for example, an increase in lung weight, correlated well with the simultaneously occurring influx of macrophages, neutrophils and lymphocytes, triggered by an earlier (day seven) release of chemokines, for example the neutrophil attractant KC (CXCL1). Considering further that pharmacological inhibition of TGFβ1 signaling from day seven was sufficient to prevent fibrosis development, but did hardly attenuate immune cell influx, our results rather suggest that presence of both, inflammation (which can be triggered by sole TGFβ1 overexpression) and a simultaneous, continuous stimulus by TGFβ1 must be initially present in order to induce fibrogenesis. This assumption is further supported by the finding that Bleomycin-induced fibrosis can be blocked by interfering with the early inflammatory reaction in this model (235).

However, another level of complexity is added to this issue by the observation that despite successful pharmacological blockage of most fibrosis-associated effects/readouts (lung weight, Ashcroft score, fibrosis assessed by µCT) but not immune cell influx, lung function remained significantly compromised. This suggests that early impairment of alveolar function must occur, potentially triggered by immune cells and associated oxidative stress, promotion of AEC apoptosis, hyperplasia, transition to a mesenchymal phenotype and/or downregulation of surfactant production. Notably, in both Bleomycin and AAV-TGFβ1-induced fibrosis models, gene expression of surfactant protein A-C was increasingly downregulated over time, which could indicate both, sole transcriptional effects and loss of AEC II cells. Nevertheless, considering that the animals that had received the ALK5 inhibitor normally gained in weight and that some of the animals that initially lost weight upon Bleomycin-mediated injury started to recover at late time points in spite of severely impaired lung function, it seems that even in absence of a strong, immediate improvement in lung function, overall disease severity can be alleviated. Finally, our ALK5 inhibition experiment also provides valuable information on the sensitivity of TGFβ1-mediated effects, as fibrosis could be largely blocked, whereas immune cell influx was mostly unaltered. These findings indicate that residual TGFβ1 expressed prior to pharmacological intervention was sufficient to induce immune cell influx, which is conceivable, given that femtomolar TGFβ1 concentrations were sufficient to mobilize immune cells in vitro (236).

Oxidative damage to DNA and RNA in endothelial and alveolar epithelial cells and subsequent cell death is believed to be a major cause of acute lung injury, thereby initiating a wounding response that includes the classical clotting cascade and inflammation. As evident from body weight tracking in our mouse experiments, Bleomycin-mediated injury resulted in a sudden decrease in body weight from day three, with a more heterogeneous course from day seven onwards with some animals continuously losing weight and other animals showing trends towards recovery.
Notably, however, a reoccurring increase in body weight was rarely associated with less severe fibrosis as assessed by other readouts, suggesting that in addition to the degree of injury, individual susceptibility may impact on the overall tolerance of Bleomycin-induced fibrosis. Similar findings in other studies contribute to the general controversy on whether Bleomycin-induced fibrosis is persistent or not (188)(237)(129). In contrast, all AAV-TGFβ1-treated animals continuously lost weight, albeit only starting from day eight after vector administration. The different kinetics are most likely a result of the different modes of disease induction, i.e. acute lung injury versus overexpression of a disease-driving transgene. For AAV-mediated transgene expression to occur, vector-transduced cells have to first synthesize double-stranded, transcriptionally active DNA from the single-stranded AAV vector genome. Additional time is also required for TGFβ1 protein expression and accumulation. Of note is, however, that both models ultimately resulted in a similar phenotype from day 21 onwards, which is reflected by a similar amount and type of immune cells and a very similar degree of ECM deposition (as evident from an increase in lung weight) and impairment of lung function.

The phenotypic changes were also nicely reflected by the transcriptional changes unraveled by next generation sequencing, which, in conjunction with pathway enrichment analyses, clearly mirrored different disease phases and processes, including inflammation, wounding and fibrogenesis. Specifically, the analysis showed that the early inflammation observed mainly at day three in the Bleomycin model was due to wounding, which involved the activation of the clotting cascade and also showed a clear type I interferon-signaling signature. Type I interferon signaling has been previously observed in bleomycin studies and was further implicated in systemic sclerosis (238)(239). Notably, a link between DNA damage and subsequent interferon signaling has been recently established, which likely explains the activation of this process upon Bleomycin instillation (240)(241). At day seven in the Bleomycin model and day 14 in the AAV-TGFβ1 model, similar immune cell influx was observed, which also occurred simultaneously to initial fibrotic changes, as suggested by an increase in lung weight. The presence of similar events was supported by the identification of cell division and proliferation-associated processes at the respective time points in both models. Furthermore, a strikingly big overlap of the gene expression changes in both models during the phase of established fibrosis at day 21 was observed. The various attempts pursued to shed light on the processes exclusively active in one or the other model was complicated by the fact that only few significantly enriched pathways were identified using the lists of exclusively altered genes. Nevertheless, clear enrichment of interferon and TLR-signaling was observed exclusively for the Bleomycin model, further supporting the assumption that the initial damage to cells and DNA most likely represents the major difference between the models. In contrast, cell cycle- and cell proliferation-associated processes were
particularly strongly enriched in the AAV model, which is conceivable, given that TGFβ1 is a major regulator of cell cycle progression (242). Although in both models immunoglobulin domain-encoding genes were strongly enriched among the top upregulated genes, this effect was particularly evident in the Bleomycin model. Unfortunately, the nature and origin of the antibody response cannot be traced back; however, given that DNA-sensing responses including TLR-signaling were found activated, these findings might point towards an auto-immune reaction triggered by release of cellular DNA, recognition of DAMPs by dendritic cells and subsequent activation of B-cells.

The finding that there is significant overlap between both models during the phase of fibrosis despite the completely different modes of disease induction, led to the hypothesis that certain processes and pathways might be central for fibrosis development and maintenance, independent of whether they are induced by injury or in a transgene-mediated fashion. A list of 1875 genes, whose expression was similarly altered during phases of fibrogenesis and fibrosis maintenance in both models was obtained. By including genes altered at day 14 and/or day 21 in the Bleomycin model and day 21 in the AAV model, it was taken into account that Bleomycin-induced fibrosis showed an earlier onset than the AAV-TGFβ1 model. While this list contained a large number of genes that have been found to be associated with fibrosis in previous studies (243)(238)(244)(205)(237), thereby also serving as a benchmark for our experimental strategy and way of analysis, it also represents a valuable repertoire for the identification of novel potential regulators, biomarkers and potential drug targets. Selected examples of particularly interesting candidates include LOXL2, HAS2, FMOD, P4HA3 and CTHRC1.

Lysyl oxidase-like 2 (LOXL2) is an enzyme with both, extracellular and intracellular functions. Extracellularly, it crosslinks collagen and elastin fibrils, thereby contributing to ECM stiffness, whereas within cells, LOXL2 was implicated in EMT promotion by deaminating a lysine residue on histone H3 and/or stabilizing SNAIL1, a transcription factor that suppresses expression of E-cadherin (206). Our lab further demonstrated prominent upregulation of LOXL2 in human IPF samples and found a role for LOXL2 in promoting FMT (data not shown). An allosteric anti-LOXL2 antibody is currently being developed for clinical testing in IPF and cancer (245). Hyaluronan synthase 2 (HAS2) is rapidly induced by TGFβ1 (246) and catalyzes an essential step during the synthesis of hyaluronan, which is an extracellular polysaccharide and important constituent of the ECM and provisional wound healing matrix. By binding to CD44, hyaluronan further has an important role in homing and adhesion of lymphocytes and promoting fibroblast proliferation and invasiveness (207). By modulating TGFβ signaling, it was further implicated in maintaining a myofibroblast phenotype and to control the deposition of collagen and fibronectin (247). Finally,
mesenchymal cell-specific deletion of HAS2 prevented the development of lung fibrosis in mice (208). Given that HAS2 is also distinctly upregulated in human IPF (GSE52463 (244), GSE32537 (248)), the data suggest that targeting HAS2 could be a strategy to interfere with both, ECM stiffening and inflammation. Fibromodulin (FMOD) is a small proteoglycan that supposedly binds TGFβ1 and that is involved in collagen assembly and structural integrity of the ECM (209). One study also suggests that FMOD knockout in mice attenuates experimental liver fibrosis (209). Prolyl 4-hydroxylase, α polypeptide III (P4HA3) is a component of collagen prolyl hydroxylase, an enzyme essential for the synthesis and proper folding of collagen. One very recent study demonstrated that pharmacological inhibition of P4HA3 attenuated collagen deposition in fibroblast in vitro and the Bleomycin model in mice, thereby reducing fibrosis-associated mortality in these animals (249). Moreover, this study also reported elevated levels of P4HA3 in the fibroblast foci of human IPF lung tissue samples. Further investigation of FMOD and P4HA3 should therefore be undertaken to explore their potential as drug targets for the treatment of pulmonary fibrosis. Collagen triple helix repeat-containing 1 (CTHRC1) was recently shown to be induced by TGFβ1 and to directly inhibit its signaling activity, supposedly by accelerating the proteasomal degradation of phospho-SMAD3, thereby representing a negative feedback loop that was also shown to suppress collagen expression (250). Furthermore, combined measurement of CTHRC1 gene expression and lung function allowed discriminating between IPF patients and healthy controls, thereby proposing CTHRC1 as a novel biomarker of pulmonary fibrosis (205).

Finally, protein-protein interaction predictions revealed the SRC kinase family members SRC, FYN and LYN as well as EGFR, PRKCA and PRKCB as the most strongly enriched interaction partners of the commonly altered genes/proteins in our models. Notably, SRC kinase and EGFR signaling are strongly implicated in pulmonary fibrosis and have been a focus of fibrosis research for many years. SRC kinases are involved in the signal cascades of several growth factor receptors, including PDGFR – which together with VEGFR and FGFR represents one of the targets of Nintedanib – thereby integrating and modulating a network of downstream effects (251). Interestingly, Nintedanib was also shown to inhibit LYN (252). SRC kinases are induced by TGFβ1 and their inhibition has proven useful in attenuating myofibroblast deposition in animal studies (253). EGFR signaling one the one hand promotes epithelial cell survival but on the other hand also induces fibroblast proliferation and ECM production. Despite these seemingly conflicting properties, inhibition of EGFR signaling has mostly proven beneficial in alleviating experimental lung fibrosis (251). Protein kinase C α and β (PRKCA/B) have been identified in fibrotic disorders of multiple organs, however, due to their broad involvement in several signaling cascades and probably cell-type specific effects, their role in pulmonary fibrosis remains largely unknown and demands further investigation.
In addition to mRNA profiling, we also analyzed the differential expression of miRNAs upon AAV-TGFβ1 and Bleomycin administration, thereby revealing a significant overlap of 166 commonly deregulated miRNAs on day 21 in both models. Similar to our findings for mRNA, the common miRNAs showed very good correlation across the models. In addition to exploring lists of the most distinctly deregulated miRNAs, we also pursued a novel approach to identify functionally relevant miRNAs by calculating correlations and anti-correlations between the temporal pattern of miRNA expression and the decrease in lung function observed in both fibrosis models. The identification of three of the most prominent fibrosis-associated miRNAs, i.e. miR-21a-5p and miR-199a-5p as the two top anti-correlated hits (i.e. upregulated in parallel to the decrease in lung function) and miR-29a-3p (i.e. downregulated in parallel to the decrease) among the top correlated hits, nicely confirmed the validity of this approach. This approach uncovered several other miRNAs that could be of interest for closer investigation. Briefly, among the upregulated, anti-correlated miRNAs, miR-132-3p was identified, which was reported to be induced by Angiotensin II and proposed to target MMP9 (254), thus possibly exhibiting anti-fibrotic properties (255). MicroRNA 335-3p was described to prevent the activation of hepatic stellate cells (i.e. the major liver fibrosis-associated cell type) by inhibiting the ECM-glycoprotein tenascin C (256). Similarly, miR-214 was shown to inhibit hepatic stellate cell activation by targeting connective tissue growth factor and was further proposed as a serum biomarker for liver fibrosis (257). Among the downregulated, lung function-correlated miRNAs, we identified miR-200a, which has been previously reported to be downregulated in models of pulmonary fibrosis and human IPF. This study suggested that miR-200 blocks TGFβ1-induced EMT and that its downregulation during fibrosis might contribute to the increase in fibroblasts (258). A particularly interesting microRNA was miR-181a-5p, as it putatively targets a lot of ECM- and fibrosis-associated proteins, including elastin, several collagen family members, PAI-1, LOX, TGFβ receptor type I and integrin β8. The top lung function-correlated miR-676-3p putatively targets collagen 11α1 and HAS2, which – as described in detail before – represents a novel, pro-fibrotic protein, whose proposed involvement in pulmonary fibrosis renders it a promising candidate for further exploration as a drug target. Interestingly, neither miR-181a-5p, nor miR-676-3p have been described in the context of pulmonary fibrosis so far. Thus, as for miR-192-5p, which was found to possibly target the pro-fibrotic enzyme LOXL2, it should be investigated, whether overexpression of these miRNAs ameliorates pathological features of pulmonary fibrosis. The use of miRNAs as therapeutics is a topic of extensive research, where basically two opposing strategies are pursued: First, delivery of miRNA-mimics to suppress mRNA targets; second, delivery of antagonomers or miRNA-binding site-harboring “sponge” constructs to antagonize the effect of upregulated miRNAs. In fact, a miR-29 mimic successfully prevented and partly reversed pulmonary fibrosis in the Bleomycin model of fibrosis (217).
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However, although the miRNA was coupled to cholesterol to facilitate stability and cellular uptake, this study clearly demonstrated that stability of miRNAs is one of several major hurdles in bringing miRNA therapy to the clinic. Besides the use of chemical modifications, using delivery vehicles such as natural exosomes or utilizing AAV-mediated miRNA expression are among the most promising approaches to improve specificity and stability (259).

In general, our strategy to correlate expression values to lung function could also be extended to other functional readouts, such as immune cell influx, protein levels of specific genes of interest or vascular leakage. Moreover, the overall strategy would also be applicable to the mRNA data set. One limitation of this approach is that by selecting for perfectly correlating or anti-correlating candidates, there is a bias for first-order interaction partners, whereas indirect regulators that might show a differing expression profile might not be identified. Therefore, to further optimize the selection strategy, we recently built a complex network database comprising mRNA and miRNA expression values, functional readouts, proposed and validated miRNA-mRNA as well as protein-protein interactions and the information on how often (at how many time points, in one or in both models?) a certain relation is given. Further middle-term goals would be to also integrate human IPF gene expression and/or proteomics data. Given that post-transcriptional regulation (except from mRNA-degradation) is not reflected by transcriptional data, the integration of proteomics would be a particularly valuable step to further optimize the transcriptome-phenotype link and feasibility has been recently shown using the Bleomycin model (237). Using this network biology approach will likely help identifying novel regulators and previously unrecognized pathways that are co-regulated with currently known key disease-driving signal cascades (260).

Taken together, our results demonstrate that sole overexpression of TGFβ1 induced pulmonary fibrosis at similar complexity as Bleomycin, underscoring the importance of TGFβ1 as a major cytokine driving this disease. The main difference between both models seems to lie in acute inflammation and wounding that transits into fibrosis in the Bleomycin model as opposed to a temporally simultaneously occurring inflammatory and fibrotic response in the AAV-TGFβ1 model, suggesting that compound testing in the AAV model might be less prone to yielding false-positive, primarily anti-inflammatory hits that ultimately fail to show efficacy under fibrotic conditions. Finally, combined mRNA and miRNA expression profiling provided novel insight into the complex nature of regulatory events driving pulmonary fibrosis and further identified several interesting protein and miRNA candidates that might be of interest for drug discovery research, including HAS2, LOXL2, FMOD as well as miR-181a-5p, miR-676-3p and miR-192-5p.
3.3 Current and future applications of AAV-riboswitch vectors

While producing AAV-TGFβ1 and other vectors for disease modeling and protein characterization purposes, we realized that the efficient production of AAV vectors harboring CMV-driven transgenes can be impeded by downstream effects triggered by the unnecessarily expressed transgenes during AAV production in HEK-293 cells. However, to enable broader application of AAV vectors in both, research and gene therapy, high vector titers are desired regardless of the transgene used. The innovative approach of integrating an artificial, guanine-responsive, self-cleaving riboswitch (GuaM8HDV) into the AAV expression cassette enabled efficient suppression of transgene expression by a single addition of guanine to the culture medium, which translated into two- to 23-fold increased AAV vector yields. Interestingly, the extent of vector increase seemed to be dependent on the severity of initial transgene toxicity. While BAX, TGFβ1 and LOXL2 transgenes severely reduced AAV yields when they were normally expressed, their suppression was found to result in approximately four- to 23-fold higher titers upon riboswitch activation. In contrast, TNFα and GFP vector production was only weakly impaired, which in turn only resulted in an about two-fold increase upon transgene suppression. Nevertheless, these results suggest that even the attenuation of moderate toxicity, including high utilization of the protein synthesis machinery, might be beneficial for AAV vector production. Importantly, our finding that the 3'-GuaM8HDV-harboring AAV-TGFβ1 remained capable of inducing pulmonary fibrosis in mice, demonstrated that such vectors can be used in vivo in preclinical research and supports further exploration of this technology for clinical use. Still, the question why transgene expression mediated by the riboswitch vector in vivo was slightly lower than with the conventional AAV remains an open question. Possible reasons include basal riboswitch activity in the absence of ligand, which had been previously observed in our initial in vitro analysis, interaction with cellular RNA-binding proteins or endogenously present guanine, albeit the latter is unlikely given that micromolar concentrations were necessary for switch activation in vitro.

With regard to riboswitch vector design, incorporation of the GuaM8HDV riboswitch into the 3' UTR of an AAV vector allowed for efficient guanine dose-dependent regulation of transgene expression, which is in line with the original publication (224). In contrast, 5'-positioning of the same riboswitch resulted in a strong decrease of transgene expression independent of guanine addition. This is most likely due to the fact that the GuaM8HDV switch contains four artificial start codons that reduce translation from the proper GFP start codon following further downstream (224). Previous work suggests that removal of these start codons could improve switching performance at the 5' position (261). Notably, in our study the exact position of the switch has not been optimized, which might explain why the switching ratio of up to 29.5 (224) was not
reproducible, thereby supporting the notion that position-based effects might be crucial for structural integrity and optimal switch performance (262). In this regard, the exceptional structural stability of HDV-derived aptazymes (224) might offer a particular advantage for their use in technological applications such as AAV production. Besides optimization of the position, a further strategy to increase the switching efficiency might be to integrate multiple copies of GuaM8HDV, as previously reported for other aptazymes (263)(264). Moreover, results obtained with constitutively active ribozymes show that expression can be changed by more than 100-fold (263), demonstrating the principle power of riboswitches, which might be achieved with novel screening strategies and further structural engineering in the future. Nevertheless, our results suggest that the degree of transgene suppression achieved using our current approach can be sufficient to fully block functional outcomes such as toxicity and other cell integrity-impairing effects. In fact, for all tested genes except from BAX, AAV titers could be increased to the yield range of AAV-GFP control vectors. However, highly potent toxic transgenes might require the use of alternative gene regulation systems, such as classical transcription factor- and dimerizer-inducible promoters (for example, tetracyclin- and rapamycin-dependent systems (265)(266)) or siRNA-mediated gene knockdown. In addition, expression might be attenuated or prevented by using tissue-specific promoters that are inactive in HEK-293 cells. However, strong ubiquitous expression as mediated by the CMV promoter has been used in the majority of viral vector-based studies and is desired in many cases, for example for the broad transduction of whole organs consisting of many different cell types like the lung and the brain, for studying gene function in multiple tissues of interest, or in therapeutic applications, where transcriptional targeting is difficult or impossible, for instance oncolytic therapy, thereby complicating this approach. A further possibility to circumvent problems arising from CMV-driven transgene expression would be the use of the SF9 insect cell AAV production system, which is well-established and in which most mammalian promoters are inactive (40). However, given that this system requires the relatively time-consuming preparation of bacmids and baculoviruses prior to actual AAV production, the HEK-293 system remains preferred for most AAV applications in basic research.

Finally, several properties of riboswitches render them advantageous in comparison to other gene regulation systems, thereby further supporting their development and application: First, aptazymes do not rely on the additional expression of transcription factors or RNA, but can be controlled by simple addition of a small molecule to the culture medium during the routine medium exchange step. Second, due to its RNA-intrinsic mode of action (i.e. mRNA destabilization and inhibition of translation), transgene regulation is independent of protein-protein or protein-DNA interactions. This is a particular advantage given that stoichiometric imbalance between protein and DNA might be responsible for the loss of regulation with inducible promoters at high
copy numbers (267). Third, as shown by our experiments, small molecule-mediated regulation allows for immediate regulation that can be dynamically reversed by replacing the medium. Fourth, as the switch sequence is largely independent of the promoters and poly(A) sequences used, it can be rapidly adapted for the regulated expression of different target gene. Fifth, the riboswitch sequence only occupies about 100 nucleotides of plasmid space, which is particularly advantageous for AAV vectors, due to their packaging limit of only 4.7 kb of DNA (221).

In addition to the results obtained in the context of AAV production optimization, we also assessed the potential of riboswitch vectors for the control of transgene expression upon gene transfer by AAV. Our finding that AAV-mediated GFP expression in HEK-293 cells can be dynamically regulated by the addition or depletion of exogenous guanine, illustrates the principle idea of using riboswitches as a gene regulation system for the use in viral vector gene therapy. Moreover, the current system might also be used to study the effects of transient gene expression changes in cellular systems. Whether gene switching is feasible in vivo has yet to be determined, however, one problem with our current riboswitch choice might be that rather high guanine concentrations were required in vitro, which likely limits in vivo applicability with regard to pharmacokinetics issues. The problem of high ligand doses together with high basal riboswitch activity and suboptimal ON/OFF ratios (including both, the problem of “leakiness” and insufficient dynamic ranges), until now limited successful in vivo application, which is why only two successful studies in mice were reported so far (262)(264). Transferring aptazymes identified by screening approaches in vitro, in bacteria or Saccharomyces cerevisiae to higher-order eukaryotes seems to represent an additional challenge in this context (268). However, advanced strategies combining rational in silico design and optimized screening platforms should in the future lead to the discovery of novel switches with improved functionality in vivo (269)(270). Although clinical proof-of-concept is still lacking, the principle of aptazyme-mediated control of viral replication and infectivity has been recently demonstrated in vitro (272). With respect to gene therapy, riboswitches possess two additional advantageous properties: First, due to their RNA-intrinsic mode of action, they are likely to behave non-immunogenic. Second, riboswitch activity can in principle be engineered to respond to small molecule drugs with preferred pharmacological properties. By altering the aptamer binding domain, several studies have proven this principle, for instance to obtain switches responsive to antibiotics (271)(273) or to respond to consciously selected, largely inert compounds, such as additives of cosmetics (274). Furthermore, a long term goal could be the development of aptazymes that specifically sense disease biomarkers such as proteins, small RNAs (275), including miRNAs or chemical metabolites and respond by inducing/blocking therapeutic gene expression according to current disease status.
Discussion

Taken together, our results suggest that riboswitches are promising tools for the targeted, dynamic regulation of gene expression in conjunction with AAV vectors, thereby bearing the potential for multiple use in technological applications (for instance, AAV production), basic research (to study effects of dynamic gene expression changes) and ultimately, as a control element for AAV-mediated expression of therapeutic transgenes.

3.4 Conclusion

In summary, the research presented in this thesis describes AAV6.2 as a powerful tool for the setup of lung disease models in mice as well as in vivo target validation and target identification studies. Based on the finding that – in contrast to a commonly used, second-generation Adenovirus-5 vector – AAV6.2 did not induce any measurable acute immune responses, we suggest AAV6.2 as the preferred vector tool for use in preclinical respiratory research. Thorough phenotypic and next generation sequencing-based characterization of a newly established AAV6.2-TGFβ1-induced model of pulmonary fibrosis in direct comparison with Bleomycin-induced fibrosis further demonstrated the value of the AAV approach for the delineation of critical disease pathways and regulators, including miRNAs. The integration of combined mRNA/miRNA profiling, functional data and protein-protein as well as protein-RNA interactions into biocomputational networks will facilitate discovery of new regulatory networks in the future. Moreover, our finding that it is feasible to pharmacologically block TGFβ1-induced fibrosis, builds the basis for the testing of drug candidates in AAV-mediated lung disease models. Finally, by integrating an artificial riboswitch into standard AAV vectors, an innovative gene regulation system was established that enables high-titer vector production in a simple, largely transgene-independent fashion. Finally, our finding that AAV-mediated transgene expression can be regulated by vector-integrated riboswitches post transduction further opens up new avenues for the exploration of this technology in the context of gene therapy.

In conclusion, based on its efficiency, low immunogenicity and proven suitability for disease modeling, we propose AAV6.2 vectors as preferred tools for a relatively easy, secure and defined setup of functional preclinical studies in the context of respiratory diseases in mice. By using riboswitch-vectors, which have proven functional in vivo, wider application of AAV vectors should become feasible.
Discussion
Materials & Methods

Modeling pulmonary fibrosis by AAV-mediated TGFβ1 expression – a proof of concept study for AAV-based disease modeling and riboswitch-controlled vector production
## 4.1 Equipment, chemicals and consumables

Table 6: Equipment used in this work

<table>
<thead>
<tr>
<th>Equipment type / purpose</th>
<th>Product name</th>
<th>Manufacturer</th>
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</thead>
<tbody>
<tr>
<td>Cell culture CO(_2) incubators</td>
<td>Heracell 240i, New Brunswick Galaxy 145</td>
<td>Thermo Fisher, Eppendorf</td>
</tr>
<tr>
<td>Cell counter</td>
<td>Countess automated cell counter</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>Microscopes</td>
<td>Axiovert 135, Axio Imager A1, EVOS FL</td>
<td>Carl Zeiss, Advanced Microscopy Group</td>
</tr>
<tr>
<td>High-content imaging device</td>
<td>IN Cell Analyzer 2000</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Electroporator</td>
<td>Eporator</td>
<td>Eppendorf</td>
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<tr>
<td>Bacteria incubator</td>
<td>BD 53</td>
<td>Binder</td>
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<tr>
<td>Bacteria shaking incubator</td>
<td>New Brunswick Innova 44</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>Western Blot device</td>
<td>iBlot Dry Blotting System</td>
<td>Life Technologies</td>
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<tr>
<td>Western Blot documentation</td>
<td>Fusion-FX7</td>
<td>Vilber Lourmat</td>
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<tr>
<td>Gel documentation</td>
<td>E-Box</td>
<td>Vilber Lourmat</td>
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<td>Flow cytometers</td>
<td>FACScan II LSR II</td>
<td>BD Biosciences, BD Biosciences</td>
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<td>Hematology Analyzer</td>
<td>Sysmex XT1800 i Vet cell analyzer</td>
<td>Sysmex</td>
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<td>Microplate reader</td>
<td>Synergy HT</td>
<td>BioTek Instruments</td>
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<td>(absorbance, fluorescence)</td>
<td>SECTOR Imager 6000</td>
<td>Meso Scale Discovery</td>
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<tr>
<td>Multiplex ELISA reader</td>
<td>NanoDrop ND-1000</td>
<td>Peqlab</td>
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<td>DNA conc. determination</td>
<td>Take3 plate/Synergy HT</td>
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<td>RNA conc. measurement</td>
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<td>Agilent Technologies</td>
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<td>RNA analyzer</td>
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<td>Eppendorf</td>
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<td>Multifuge X3R</td>
<td>Thermo Fisher, Kendro Laboratory Products</td>
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<td>Ultracentrifuge</td>
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<td>Kendro Laboratory Products</td>
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<td>Tissue homogenator</td>
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<td>HM 3555 Rotary Microtome</td>
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<td>BOND-MAX stainer</td>
<td>Leica Biosystems</td>
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<td>Varistain Gemini ES</td>
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<td>Perkin Elmer</td>
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<td>Lung function measurement</td>
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<td>Scireq</td>
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<td>Reference, Research Plus, Pipet-lite</td>
<td>Eppendorf, Rainin</td>
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<td>E1-ClipTip Multichannel Pipettes</td>
<td>Thermo Fisher</td>
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<td>Dispenser pipette</td>
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Table 7: Chemicals and substances used in this work

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<td>β-Mercaptoethanol</td>
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<td>Bovine serum albumin</td>
<td>Serva Electrophoresis</td>
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<tr>
<td>CaCl₂ dihydrate</td>
<td>Sigma-Aldrich</td>
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<td>Chloroform</td>
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<td>CsCl</td>
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<td>EDTA disodium salt</td>
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<td>Ethidium bromide solution</td>
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<td>Formaldehyde solution, 37 %</td>
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<td>Glycerol</td>
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<td>G5516</td>
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<td>Guanine</td>
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<td>HCl solution, 1 M</td>
<td>Sigma-Aldrich</td>
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<td>HEPES</td>
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<td>Hoechst 33342</td>
<td>Thermo Fisher</td>
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<td>KCI</td>
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<td>MgCl₂ solution, 1 M</td>
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<td>Na₂HPO₄ dihydrate</td>
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<td>NaCl</td>
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<td>NaHCO₃ solution, 7.5 %</td>
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<td>NaOH solution, 4 M</td>
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<td>Paraformaldehyde, 4 %</td>
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<td>TRIS</td>
<td>Carl Roth</td>
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<td>Triton X-100</td>
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<td>Xylene</td>
<td>Sigma-Aldrich</td>
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Materials & Methods

Table 8: Consumables used in this work

<table>
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<tr>
<th>Consumable</th>
<th>Product name</th>
<th>Manufacturer, cat. no.</th>
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<td>Cell culture flasks</td>
<td>TC Flask T175, Stand., Vent. Cap</td>
<td>Sarstedt, #83.3912.002</td>
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<tr>
<td>Cell culture dish, 15 cm</td>
<td>TC Dish 150, Standard</td>
<td>Sarstedt, #83.3903</td>
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<td>Cell culture multiwell plates</td>
<td>TC Plate, 6 well, Standard, F</td>
<td>Sarstedt, #83.3920</td>
</tr>
<tr>
<td>Cell culture multiwell plates</td>
<td>TC Plate, 24 well, Standard, F</td>
<td>Sarstedt, #83.3922</td>
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<tr>
<td>Cell culture multiwell plates</td>
<td>TC Plate, 96 well, Standard, F</td>
<td>Sarstedt, #83.3924</td>
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<td>Collagen-coated 24-well plate</td>
<td>BioCoat collagen I 24-well clear TC</td>
<td>Corning, #356408</td>
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<tr>
<td>Cell culture 24-well plate, black</td>
<td>Black visiplate TC</td>
<td>Wallack, #1450-605</td>
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<td>Cell scraper</td>
<td>Disposable cell scraper</td>
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<td>Sarstedt, #62.554.502</td>
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<tr>
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<td>Tube 50 mL, 114x28 mm, PP</td>
<td>Sarstedt, #62.547.254</td>
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<td>Conical tubes</td>
<td>Falcon 225mL PP CentrifugeTube</td>
<td>Corning, #352075</td>
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<tr>
<td>Reaction tubes (0.5-5 mL)</td>
<td>Safe-Lock tubes</td>
<td>Eppendorf</td>
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</tbody>
</table>

Details on further consumables, kits and reagents are given at the relevant position within the respective methods description in the following paragraphs.

4.2 Molecular biologic, cell biologic and biochemical methods

4.2.1 Cell culture

HEK-293-H (henceforth referred to as HEK-293) cells (#11631-017, Thermo Fisher, Waltham, MA, USA) and A549 cells (#86012804, Sigma-Aldrich, St. Louis, MO, USA) were cultured in DMEM medium supplemented with glucose, GlutaMAX and pyruvate (#31966-021, Gibco/Thermo Fisher) and 10 % FCS (#10500064, Lot#07Q0027K, Gibco/Thermo Fisher). NCI-H292 cells (#CRL-1848, ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with GlutaMAX, HEPES (#72400-021, Gibco/Thermo Fisher) and 10 % FCS. Normal human lung fibroblasts (#CC-2512, Lonza, Walkersville, MD, USA) were cultured in Fibroblast Basal Medium (FBM) supplemented with insulin, rhFGF-B and FCS (FGM-2 BulletKit, #CC-3132, Lonza). All cells were grown in an incubator at 37 °C, 5 % CO₂ and 95 % relative humidity. For subculturing, cells were washed with 1x PBS and detached using trypsin-EDTA (#T4049, Sigma-Aldrich). After addition of fresh medium, cells were pelleted by centrifugation at 200 xg for 3-5 min, resuspended in fresh medium, counted and seeded in new flasks/plates/dishes at the desired cell number or following a defined split ratio.
4.2.2 Transfection and transduction of cells

Unless stated differently, cells were seeded to reach about 70-80 % confluence at the day of transfection/transduction. Table 9 shows the cell numbers used for different cell lines, culture scales, seeding time points and transfection methods. HEK-293 cells were routinely transfected using the calcium-phosphate procedure. Briefly, 0.5 µg total DNA per cm² of culture area were mixed with 1/10 culture volume of 300 mM CaCl₂ and added dropwise to an equal volume of 2x HBS buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na₂HPO₄). After incubation at RT for 2 min, the mix was added to the cells. After 5-6 h of incubation, the culture medium was replaced by fresh medium. In the riboswitch experiments, the fresh medium was optionally supplemented with guanine, which was previously dissolved in 0.1 M NaOH to a stock concentration of 20 mM. For A549 cell transfection, 0.5 µg DNA in 25 µL Opti-MEM (#11058021, Life technologies) were mixed with 1 µL lipofectamine-2000 (#11668027, Life technologies) in 25 µL Opti-MEM, and 50 µL of this mix were added per 24-well. The culture medium was replaced 4-5 h later. For the transduction of NCI-H292 and NHLF cells, recombinant AAV vectors were added directly to the culture medium at the desired multiplicity of infection (MOI, i.e. AAV vector genomes per seeded cell). In this case, no medium change was performed.

4.2.3 Cloning of expression constructs

The coding sequences flanked with appropriate restriction sites were synthesized at GeneArt (Life technologies) or amplified from plasmids available in the lab. For the generation of pAAV-CMV-MCS-SV40, the SV40 polyA sequence was PCR-amplified from a plasmid present in our lab. For this purpose, forward (ccgagatcttctgttattgcagcttatg) and reverse primers (cggcggtccgtttcacgtgcgcgcttaaaagga) harboring BglII and PmlI restriction sites (underlined), respectively, were synthesized. The PCR reaction was set up as follows: 50 ng plasmid DNA template, 200 µM dNTPs, 0.5 µM of each primer, 1x Phusion HF Buffer and 1 U Phusion HF DNA Polymerase (#M0530, New England BioLabs, Ipswich, MA, USA). PCR program: 2 min at 98 °C, 30 cycles of 30 sec at 98°C, 30 sec at 60 °C (lowest primer melting temperature), 50 sec at 72 °C (20 sec/1000 kb amplicon), final elongation 7 min at 72 °C.

For subcloning, the starting plasmids were digested using appropriate “Fast Digest” restriction enzymes (Thermo Fisher) and applied to preparative DNA gel electrophoresis to separate the cloning fragments of interest, which were subsequently purified from the excised agarose gel pieces (NucleoSpin Gel and PCR Clean-up, #740609, Macherey-Nagel, Düren). Target vector backbone DNA (50 ng) and insert fragments were ligated at a molar ratio (vector : insert) of 3 : 1 using the T4 DNA ligase (#EL0016, Thermo Fisher) at 22 °C for 1 h. To deplete salt, the ligation
reaction was purified using the DNA Clean & Concentrator-5 kit (#D4004, Zymo Research, Irvine, CA, USA) and the eluted DNA was used to transform ElectroMAX DH5α-E Competent *E.coli* (#11319-019, Life technologies). Transformed bacteria were plated on 100 µg/mL Ampicillin-containing LB-Agar plates (LB-Agar, #X969.1, Carl Roth, Karlsruhe) at different dilutions and grown at 37 °C overnight. Several single colonies were picked and used to inoculate 2 mL LB-Amp (100 µg/ml; LB medium, #X968.1, Carl Roth) mini cultures, which were grown in a shaker incubator at 37 °C and 160 rpm overnight. Plasmid DNA was prepared using the NucleoSpin Plasmid kit (#740588, Macherey-Nagel) and positive clones were detected by confirming expected DNA band patterns by specific restriction digestions. The remaining mini culture of a positive clone was used to inoculate a 300 mL LB-Amp maxi culture for plasmid amplification and after growth overnight, plasmid DNA was prepared using the NucleoBond Xtra Maxi EF kit (#740424, Macherey-Nagel). Plasmid DNA identity was finally validated by sequencing (Sequiserve GmbH, Vaterstetten).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Scale</th>
<th>Cells seeded/well</th>
<th>Time prior to transfection/treatment [h]</th>
<th>Confluence [%]</th>
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<td>2.40E+04</td>
<td>16</td>
<td>70-80</td>
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<tr>
<td></td>
<td>24-well</td>
<td>1.50E+05</td>
<td>16</td>
<td>70-80</td>
</tr>
<tr>
<td></td>
<td>6-well</td>
<td>1.00E+06</td>
<td>16</td>
<td>70-80</td>
</tr>
<tr>
<td></td>
<td>6-well</td>
<td>2.40E+05</td>
<td>72</td>
<td>70-80</td>
</tr>
<tr>
<td></td>
<td>15 cm dish</td>
<td>2.40E+06</td>
<td>72</td>
<td>70-80</td>
</tr>
<tr>
<td>A549</td>
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<td>30</td>
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<tr>
<td>NCI-H292</td>
<td>96-well</td>
<td>3.00E+04</td>
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<td>70-80</td>
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<td>NHLF</td>
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<td>70-80</td>
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<td>48-well</td>
<td>4.00E+04</td>
<td>16</td>
<td>70-80</td>
</tr>
</tbody>
</table>

### 4.2.4 Expression constructs

pAAV-RC, pHelper (AAV Helper-free system, #240071, Agilent Technologies, Waldbronn) and pDP8.ape (PlasmidFactory, Bielefeld) were purchased. All other expression constructs used in this work were generated using the cloning procedure described above and the cloning strategy described in Table 10. For the generation of pAAV-CMV-Tgfβ1-hGH, the murine Tgfβ1 cDNA was codon-usage optimized for expression in mice and synthesized at Life Technologies before being cloned into a pAAV-CMV-MCS-hGH vector. The Tgfβ1 sequence contains C223S and C225S mutations which increase the fraction of active protein (202). As a stuffer sequence for pFB-stuffer, a fragment of the 3’-UTR of the E6-AP ubiquitin-protein ligase (UBE3A; accession no.
AH006486.2, position 994..2740) was synthesized and cloned into the pFB vector. The stuffer sequence is followed by a SV40 polyA signal. Selected vector maps are depicted in Figure 40. Moreover, the DNA sequence of the functional 3’GuaM8HDV riboswitch-containing pAAV vector (Figure 40d) is shown in Figure 41.

<table>
<thead>
<tr>
<th>Plasmid name</th>
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<th>Target vector backbone origin</th>
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<th>3’ RE site</th>
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<td>-</td>
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<td>pAAV-CMV-MCS-SV40</td>
<td>BamHl</td>
<td>HindIII</td>
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<td>HindIII</td>
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<td>BamHl</td>
<td>HindIII</td>
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</table>
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Figure 40: Vector maps of selected constructs used in this work

Vector maps of (a) pAAV-stuffer, (b) pAAV-CMV-Tgfb1-hGH, (c) pAAV-CMV-eGFP-SV40 and (d) pAAV-CMV-eGFP-3’GuaM8HDV-SV40 are depicted. Amp= ampicillin, CMV= cytomegalo virus, eGFP= enhanced green fluorescent protein, Gent= gentamicin, hGH= human growth hormone, ITR= inverted terminal repeat, ori= origin of replication, Res= resistance, SV40= Simian vacuolating virus 40, Tn7= bacterial transposon Tn7, TGFβ1= transforming growth factor β1.

4.2.5 AAV production and purification

HEK-293 cells were seeded in 20 to 120 15 cm tissue culture dishes three days prior to transfection to reach 70-80 % confluence on the day of transfection (Table 9). The cells were transfected using the calcium-phosphate method as described above, where 0.5 µg total DNA per cm² of culture area were used. AAV6.2 vectors were produced by co-transfecting pAAV-AAV6.2cap, pHelper and a plasmid carrying the transgene cassette of interest flanked by AAV2 ITRs. For AAV9 production, pAAV-AAV9cap was used instead of pAAV-AAV6.2cap. In the case of AAV8, a double transfection using pDP8.ape together with the transgene plasmid was performed. Independent of the AAV capsid variant used, the co-transfected plasmids were used in an equimolar ratio. Transfected cells were then grown for 72 h and detached by either scraping using a rubber policeman or by addition of EDTA solution (6.25 mM final concentration) to the medium.
After pelleting the cells at 700 x g and RT for 10 min, they were resuspended in “lysis buffer” (50 mM Tris, 150 mM NaCl, 2 mM MgCl₂, pH 8.5). For CsCl purification, the cells of ten 15 cm dishes were dissolved in 30 mL lysis buffer. For one iodixanol gradient, the desired amount of cells (up to 40 dishes) was dissolved in 8 mL lysis buffer. Cells were then lysed by three freeze/thaw cycles using liquid nitrogen and a 37 °C water bath. For each initially transfected dish, 100 units Benzonase nuclease (#70664, Merck Millipore, Darmstadt) were added to the mix to digest genomic and plasmid DNA and incubated for 1 h at 37 °C. After pelleting cell debris for 15 min at 2500 x g, the supernatant was used for further purification.

The DNA sequence of the ITR-flanked, 3’GuaM8HDV ribo switch-containing expression cassette of pAAV-CMV-eGFP-3’GuaM8HDV-SV40 is depicted. Sequence motifs are color coded as indicated in the legend.
4.2.5.1 CsCl-based AAV purification protocol

CaCl$_2$ was added to the supernatant obtained after cell lysis and benzonase-treatment (see above) at a final concentration of 25 mM. After incubating the mix for 1 h on ice, it was centrifuged for 15 min at 2500 x g and 4°C, and the pellet was discarded. 40 % PEG-8000 was added to the supernatant to a final concentration of 8 % and the mix was incubated on ice for 3 h. After centrifugation for 30 min at 2500 x g and 4°C, the pellet was resuspended in “resuspension buffer” (50 mM HEPES, 150 mM NaCl, 25 mM EDTA, pH 7.4) and dissolved overnight at 4°C on a tube rotator. The suspension was then centrifuged for 30 min at 4°C and 2500 x g and 3.149 M CsCl were added to the supernatant (corresponding to a refractive index (RI) of 1.3710). Using 39 mL Quick-Seal tubes (#342414, Beckman Coulter, Brea, CA, USA) and a Beckman Type 70 Ti rotor, the mix was ultracentrifuged for 23 h at 63000 rpm and 21°C. The tube was then punctured at the bottom using a 20 G needle (#4657519, B. Braun, Melsungen) and 1 mL fractions were collected. All fractions within a RI range of 1.3758 and 1.3703 were pooled and applied to five rounds of dialysis against 2 L of phosphate-buffered saline (PBS) using Slide-A-Lyzer MWCO 20,000 dialysis cassettes (#66003, Thermo Fisher). The dialyzed product was finally concentrated using Amicon Ultra-15 centrifugal filter units with a MWCO of 100 kDa (#UFC910024, Merck Millipore, Billerica, MA, USA). After addition of glycerol at a final concentration of 10 %, the product was sterile filtered using the Ultrafree-CL filter tubes (#UFC40GV05, Merck Millipore), aliquoted and stored at -80°C.

4.2.5.2 Iodixanol-based AAV purification protocol

To prepare an iodixanol gradient, 60 % iodixanol (OptiPrep, #D1556, Sigma-Aldrich) was diluted to 15, 25, 40 and 58 % in PBS-MK (1x PBS, 1 mM MgCl$_2$, 2.5 mM KCl). NaCl was added to the 15 % phase at 1 M final concentration. 1.5 µL of 0.5% phenol red were added per milliliter to the 15 % and 25% iodixanol solutions and 0.5 µL were added to the 58 % phase to facilitate easier distinguishing of the phase boundaries within the gradient. 8 mL of 15 %, 6 mL of 25 %, 8 mL of 40 % and 5 mL of 58 % iodixanol solution were then successively underlaid in 39 mL Quick-Seal tubes (Beckman Coulter). The processed cell lysate (see above) was then gently overlaid onto the gradient and filled up with lysis buffer when necessary. After centrifugation in a 70 Ti rotor for 2 h at 63,000 rpm and 18 °C, the tube was punctured at the bottom using a 16 G needle (#300637, B. Braun) and 1 mL fractions were collected. For AAV collection, the first five milliliters (corresponding to the 58 % phase) were discarded, and the fractions obtained from the 40 % phase were analyzed by measuring absorbance at 20-fold dilution at 340 nm to identify the main contaminating protein peak, as previously described (42). All fractions below this peak were pooled. PBS was added to the pool to reach a total volume of 15 mL and ultrafiltered/
concentrated using Amicon Ultra-15 centrifugal filter units with a MWCO of 100 kDa (Merck Millipore). After concentration to ~1 mL, the retentate was filled up to 15 mL and concentrated again. This process was repeated until an arithmetical value of <0.1% iodixanol concentration was reached (usually 3 cycles). Glycerol was added to the preparation at a final concentration of 10%. After sterile filtration using the Ultrafree-CL filter tubes (Merck Millipore), the product was aliquoted and stored at -80°C.

4.2.6 AAV genomic titer determination

For the determination of genomic titers of purified AAV stocks, viral DNA was isolated using the Viral Xpress DNA/RNA Extraction Reagent (#3095, Millipore) and quantified by subsequent qPCR analysis using the QuantiFast Probe RT-PCR Kit (#204456, Qiagen) and primers specific for the CMV-promoter (forward: 5’-CGTCAATGGGTGGAGTATTTACG-3’, reverse: 5’-AGGTCATGTACTGGG CATAATGC-3’, probe: 5’-AGTACATCAAGTGATCATATGCGCAAAGTACGGCC-3’) or the stuffer sequence (forward: 5’-CCAACAGAACTACTCTCCAAGGA-3’, reverse: 5’-GATACAGGGACAAAAAAGTAGCACA TAC-3’, probe: 5’-TTGCCACCACCCGGAGTCACCCTTC-3’). CMV-promoter- or stuffer sequence-containing plasmids were routinely used to prepare standard curves (2.5x10^9 copies/µL, serially diluted 1:5) for quantification. For measurement, viral DNA was diluted 1:200 in water. qPCR conditions were as described below (4.2.8). For the determination of AAV titer in cell lysate, AAVs were released from the transfected cells by freezing and thawing (see AAV production and purification) and the lysate was incubated with 250 U/mL Benzonase nuclease for 24 h at 37 °C to deplete residual plasmid DNA. After pelleting cell debris at 2500 xg for 10 min, NaOH was added to 100 mM final concentration and the mix was incubated at 70 °C for 30 min to both, inactivate benzonase and lyse the AAV particles to release viral DNA. HCl was finally added to neutralize the solution. For qPCR analysis, the samples were diluted 1:100 in water.

4.2.7 RNA preparation from cells and lung tissue

Cells were lysed by removing the medium and adding RLT buffer (#79216, Qiagen, Hilden, Germany) directly to the cells. RNA was subsequently purified using RNeasy Mini or 96 kits (#74104 or #74181, Qiagen). For total lung RNA preparation, either the total or only the right lung (in cases where the left lung was used for histology) was immediately flash frozen in liquid nitrogen. Frozen lungs were homogenized in 2-4 mL precooling RLT buffer + 1 % β-mercaptoethanol using the Peqlab Precellys 24 Dual Homogenizer and 7 mL-ceramic bead tubes (#91-PCS-CK28L, Peqlab, Erlangen). 150 µL homogenate were then mixed with 550 µL QIAzol Lysis Reagent (#79306, Qiagen) in Phase Lock Gel tubes (#2302830, 5 Prime, Hilden). After addition of 140 µL chloroform, the mixture was shaken vigorously for 15 sec and centrifuged for 5 min at
12,000 xg and 4 °C. 350 µL of the upper aqueous phase (containing RNA) were then further purified using the miRNeasy 96 Kit (#217061, Qiagen) according to the manufacturer’s instructions. After purification, RNA concentration was determined using a Synergy HT multimode microplate reader and the Take3 module (BioTek Instruments, Winooski, VT, USA). RNA quality was assessed using the 2100 Bioanalyzer (Agilent Technologies).

4.2.8 Gene expression analysis by qPCR

Equal amounts of RNA (0.1-1 µg) of each sample were reversely transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (#4368814, Life technologies) according to the manufacturer’s instructions. qRT-PCR reactions were set up using the QuantiFast Probe RT-PCR Kit (#204456, Qiagen), gene specific primer/probe sets (see Table 11) and 0.4-1 µL of cDNA per 10 µL reaction. The PCR conditions were as follows: 10 min at 95°C (once), 15 sec at 95°C and 1 min at 60°C (40 cycles). Gene expression analysis was carried out using the 7900HT Fast Real-Time PCR System and the SDS 2.4 software (Applied Biosystems). Gene expression was normalized to the expression of RNA polymerase II (Polr2a) which was used as a housekeeping gene.
4.2.9 Riboswitch GFP assay

HEK-293 cells were seeded in black multiwell plates, transfected as described above and incubated for 24 to 72 h, as specified in the results section. At indicated time points, fluorescence microscopic pictures were taken using the IN Cell Analyzer 2000 at 10x magnification. For direct fluorescence measurement, cells were washed with PBS and fluorescence was measured using a Synergy HT multimode microplate reader at 488 nm (excitation) and 535 nm (emission). Fluorescence values were normalized as described below. For flow cytometric analysis, cells were washed with PBS, detached using trypsin-EDTA, washed again and resuspended in PBS containing 10 % FCS. Cells were analyzed for eGFP fluorescence using a FACSCanto II flow cytometer and data were analyzed using FACSDiva software (BD Biosciences). Mean fluorescence intensity values were recorded, the fluorescence of untreated cells was subtracted, and the resulting values were divided by the ratio of GFP-positive cells to compensate for potential differences in transfection efficiency. The corrected fluorescence values of the GFP-control construct without guanine addition was set 100 % and mean fluorescence of the riboswitch construct-transfected samples were calculated accordingly.
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### Table 11: qPCR primer/probe sets used in this work

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<td></td>
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### 4.2.10 Fibroblast-to-myofibroblast (FMT) assay

2,000 NHLF cells were seeded in black poly-D-lysine-coated 384-well plates (#6007580, PerkinElmer, Waltham, MA, USA) and cultured in FBM medium, as described above, for 24 h. The cells were then washed with 1x HBSS, 10 mM HEPES, 0.375 % NaHCO₃ and grown in serum-free FBM medium overnight. The medium was then replaced with conditioned medium or medium supplemented with recombinant TGFβ1 (#T7039, Sigma-Aldrich, 5 ng/mL) and the cells were grown for 72 h. After fixing the cells for 30 min (2 % Formaldehyde, 1 µM Hoechst 33342 in PBS), the cells were washed with PBS and permeabilized with 1 % Triton-X-100/PBS for 20 min at RT.
After further washing, cells were blocked with 2 % BSA/PBS for 30 min at RT and washed again. Following further washing, α-smooth muscle actin was stained using a monoclonal mouse anti-αSMA antibody (#A2547, Sigma-Aldrich, 1:1000 in PBS) at 37 °C for 1 h. The cells were washed and an Alexa Fluors-647-coupled secondary antibody (#A-21236, Thermo Fisher, 1:1000 in PBS) was added for 1 h at 37 °C. The stained cells were finally stored in PBS. Alpha-SMA fibers and Hoechst-stained nuclei were finally detected using an IN Cell Analyzer 2000 and a customized IN Cell Investigator software script that automatically detects and quantifies α-SMA fibers per cell.

4.2.11 Cytotoxicity assay

Cytotoxicity was assessed by measuring Lactate dehydrogenase (LDH) release in the supernatant of cultured cells using the CytoTox-ONE Homogeneous Membrane Integrity Assay (#G7890, Promega, Madison, WI, USA) according to the manufacturer’s instructions. LDH-release measured using untreated cells served as a negative control and was set to 0 %. Triton-lysed untransfected HEK-293 cells served as a positive control and were set to 100 %.

4.2.12 Lung immune cell analysis

Total bronchoalveolar lavage (BAL) cell counts were determined using the Sysmex XT1800 iVet cell analyzer. 200 µL of BAL samples were further used to prepare cytopsins for differential cell analysis by microscopic differentiation. For the identification of CD4⁺ and CD8⁺ lymphocytes, BAL samples were centrifuged at 250 xg for 7 min and the pooled cell pellets were resuspended in 2 mL ACK lysing buffer (#10-548E, Lonza, Basel, Switzerland) to lyse erythrocytes. After incubation for 5 min, cells were washed with FACS buffer (PBS, 0.1 % BSA) and 0.25 µg rat anti-mouse CD16/CD32 (#553142, BD Biosciences, Franklin Lakes, NJ, USA) were added as Fc-block and incubated for 10 min at 4 °C. Subsequently, cells were stained by adding 1 µg of either rat anti-mouse CD4-FITC (#553055, BD Biosciences) or rat anti-mouse CD8-APC (#553035, BD Biosciences) antibody for 30 min at 4°C. Cell samples stained with 1 µg of rat IgG2bκ (#553988, BD Biosciences) and rat IgG2ακ (#552784, BD Biosciences), respectively, served as isotype controls. After washing the cells, 100 µL of 4 % PFA were added for fixation. After renewed washing, cells were analyzed using a LSR II flow cytometer.

4.2.13 Total protein measurement (Bradford assay)

Total protein content in BAL or cell culture-derived samples was determined using the Pierce Coomassie Plus (Bradford) Assay Kit (#23238, Thermo Fisher) and a BSA-based standard curve.
4.2.14 Protein detection using ELISA

Protein levels in cell supernatant or BAL fluid were determined using the Human Serpin E1/PAI-1 Quantikine ELISA Kit (#DSE100, R&D Systems, Minneapolis, MN, USA), the Human TNF-alpha Quantikine ELISA Kit (#DTA00C, R&D Systems) and the Mouse TGF-beta 1 Quantikine ELISA Kit (#MB100B, R&D Systems) according to the manufacturer’s instructions. To measure total TGFβ1 (i.e. active and latent), samples were acid-activated as described in the assay manual, whereas untreated samples were used to determine TGFβ1 present in its active, mature form. BAL levels of murine IL1β, IL6, KC/Cxcl1, TNFα, IL12 and IFN-γ were measured using the Proinflammatory Panel 1 (mouse) V-PLEX Kit (#K15048D-1, Meso Scale Discovery, Rockville, MD, USA) and the SECTOR Imager 6000.

4.2.15 Protein detection using Western Blot

SDS-PAGE gels were run using NuPAGE Novex 4-12 % Bis-Tris Protein Gels (#NP0322BOX, Life technologies) and the Precision Plus Protein WesternC Marker (#161-0385, Bio-Rad, Hercules, CA, USA). Western Blotting was conducted using the iBlot Dry Blotting System according to the manufacturer’s instructions. Ponceau S staining was used to visualize all blotted protein. Immunostaining was conducted using standard methods. Briefly, the membrane was blocked using 1X TBST + 4 % skim milk powder (#15363, Merck Millipore) and incubated overnight at 4 °C with diluted primary antibody. The primary antibodies used were the rabbit anti-human BAX (#a3533, Dako, Carpinteria, CA, USA, 1:1000), polyclonal goat anti-V5 tag antibody (#ab95038, Abcam, Cambridge, UK, 1:1000) and monoclonal mouse anti-Vinculin antibody (#V9131, Sigma-Aldrich, 1:2,000). After washing (4 x 3 min with 1X TBST), a HRPO-conjugated secondary antibody was added (#111-035-003, #705-035-003, #115-035-062, all Dianova, Hamburg, all 1:10,000) and incubated at RT for 1 h. Proteins were finally visualized using the SuperSignal West Pico Chemiluminescent Substrate (#34080, Life technologies) and imaged using the Fusion FX-7 system.

4.2.16 Histological examination

For cryo samples, the lung was dissected and embedded in Tissue-Tek O.C.T. Compound (#4583, Sakura Finetek, Alphen aan den Rijn, The Netherlands) and frozen on dry ice. Using a cryomicrotome, tissue slices of 15 µm were prepared, mounted with ProLong Gold Antifade Mountant with DAPI (#P36935, Thermo Fisher) and analyzed by (fluorescence) microscopy. For FFPE samples, the trachea was cannulated (1.2 mm with Luer, Hugo Sachs Elektronik, March), the left lung lobe was separated from the right lung lobe by ligature and the right lung was cut off. The left lung lobe was then mounted to a separation funnel filled with 4 % paraformaldehyde (PFA) and inflated under 20 cm water pressure for 20 minutes. The filled lobe was then sealed by
ligature of the trachea and immersed in 4 % PFA for at least 24 h. Subsequently, PFA-fixed lungs were embedded in paraffin. Using a microtome, 3 µm lung sections were prepared, floated out into a water bath, from which the flattened section was taken up onto an object slide. After drying the sections for 24 h at 40 °C, they were deparaffinized using xylene and rehydrated in a descending ethanol series (100-70 %). When establishing new immunohistochemistry (IHC) protocols, different epitope retrieval methods were automatically performed using the BOND-MAX stainer, which compares enzymatic (Bond enzyme pretreatment kit, #35607, Leica Biosystems), pH6-citrate buffer- (Bond Epitope Retrieval solution 1, #35608, Leica Biosystems) and pH9-EDTA/heat-mediated (Bond Epitope Retrieval solution 2, #AR9640, Leica Biosystems) antigen retrieval. Masson’s trichrome staining was automatically performed using the Varistain Gemini ES Automated Slide Stainer according to established protocols (276). For GFP- and Surfactant protein C-IHC, enzymatic antigen retrieval was performed and antibodies were diluted at indicated ratios in Bond primary antibody diluent (#35089, Leica Biosystems). Slides were stained with a polyclonal rabbit anti-GFP antibody (#ab290, Abcam, Cambridge, UK, 1:1000), a polyclonal rabbit anti-SP-C antibody (#AP12333A, Abgent, San Diego, CA, USA, 1:50) or appropriate isotype control antibodies. Slides that had only received antigen retrieval served as an additional negative control. Finally, sections were mounted with Aquatex medium (#108562, Merck Millipore).

4.2.17 Ashcroft scoring

Parasagittal FFPE lung sections including a longitudinal cut of the main bronchus were assessed for their degree of lung fibrosis using the scale and grades defined by Ashcroft et al. in Table 1 of the original publication (204). To this end, the slides were systematically analyzed by microscopy at 10x magnification. Grades were assigned to individual fields of view according to the predominant degree of fibrosis that occupied more than half of the field area. Finally, the mean value of all fields was taken as the fibrosis score for the whole examined section.

4.3 In vivo methods and animal handling

4.3.1 Animals

For the comparison of Ad5- and AAV6.2-stuffer vectors, female Balb/c mice (9-12 wks. of age) were purchased (Charles River Laboratories, Sulzfeld). AAV-TGFβ1 experiments were performed using male C57Bl/6J mice (9-12 wks. of age, Charles River Laboratories). Mice were housed in 500 cm² individually ventilated cages (GM500 Sealsafe Mouse Plus cage, Tecniplast, Buguggiate, Italy)
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in groups of 4-6, with water and chow provided *ad libitum*. The cages were equipped with nesting material (paper, bedding), igloo houses, gnawing sticks and a rack for fodder, which also serves as a climbing frame. All animals were allowed to acclimate for at least 1 week before the start of experiments. All experiments were conducted in accordance with the German law on animal welfare (TierSchG) and have been approved by the Regierungspräsidium Tübingen (approval no. 12-030).

4.3.2 Viral vector/substance administration and sampling

For virus application, mice received light anesthesia (3-4 % Isoflurane, Abbott, Wiesbaden) and were put into supine position on an angled operation table. Using a 22G plastic catheter (Vasofix, #4268091B, B. Braun), the mice were administered 50 µL of viral vector suspension or PBS into the trachea. The AAV concentrations used are indicated in the results section for each of the experiments. At indicated time points, mice were either euthanized by intraperitoneal pentobarbital injection (400 mg/kg, Narcoren, Merial, Hallbergmoos) or anaesthetized and subjected to micro-CT and/or lung function measurement. Afterwards, the lung was dissected, lung weight was determined and the lung was flushed to obtain bronchoalveolar lavage (BAL) fluid. BAL samples were obtained by flushing the lungs twice with 700 µL HBSS + 0.6 µM EDTA + 1 % BSA. Finally, the lungs were either flash frozen in liquid nitrogen or processed for histology as described above.

4.3.3 Lung function measurement

For lung function measurement, mice received anesthesia by i.p. application of 60 mg/kg pentobarbital (Narcoren, Merial) and 2.9 mg/kg xylazine hydrochloride (Rompun 2%, Bayer, Monheim) in isotonic sodium chloride solution in a volume of 10 mL/kg. After cannulation of the trachea (1.2 mm with Luer, #73-2836, Hugo Sachs Elektronik, March), mice received 0.64 mg/kg pancuronium bromide (Pancuronium Inresa, Inresa, Freiburg) diluted in isotonic NaCl solution in a volume of 4 mL/kg by i.v. administration. Via the cannula, mice were connected to the flexiVent FX system, which calculates resistance, compliance and elastance from single-frequency forced oscillation technique measurements. Immediately after lung function measurement, mice were euthanized by a pentobarbital overdose (800 mg/kg, i.p.). Data were analyzed using the flexiWare 7 software (Scireq, Montreal, Canada).

4.3.4 Micro-CT analysis

Mice were anaesthetized using 4 vol. % isoflurane (Abbott, Wiesbaden) and anesthesia was maintained using 1.5 vol. % isoflurane via a nose cone during CT analysis. Mice were placed in an
animal holder in a supine position and CT scanning was performed on a Quantum FX microcomputed tomograph, using the following settings: 24 mm field of view (FOV), tube voltage 90 kV, tube current 160 µA, total acquisition time 4.5 min (retrospective respiratory and cardiac gating). After data acquisition, reconstruction automatically creates a 3D image data set with a matrix size of $512^3$ and an isotropic voxel size of 46 µm, the data of which were converted to Hounsfield Units. Segmentation of the entire lung was done manually using MicroView 2.1 (GE Healthcare, London, Canada) and transferred into a 3D-volume of interest. After histogramming, values between -150 and 100 HU were considered as fibrotic areas. The fibrotic volume was calculated by multiplying the number of values with voxel size.

4.4 Next generation sequencing, bioinformatics and statistics

4.4.1 Library preparation

cDNA libraries were prepared using the TruSeq RNA Sample Preparation Kit (#RS-122-2001, Illumina, San Diego, CA, USA). Briefly, 200 ng of total RNA (see 4.2.7) were subjected to polyA enrichment using oligo-dT-attached magnetic beads. PolyA-containing mRNAs were then fragmented into pieces of approximately 150-160 bp using heat and chaotropic salts. Following reverse transcription with random primers, the second cDNA strand was synthesized by DNA polymerase I. After an end repair process and the addition of a single adenine base, phosphothymidine-coupled indexing adapters were coupled to each cDNA, which facilitate sample binding to the sequencing flow cell and further allows for sample identification after multiplexed sequencing. Following purification and PCR enrichment of the cDNAs, the library was diluted to 2 nM and clustered on the flow cell at 9.6 pM, using the TruSeq SR Cluster Kit v3-cBot-HS (#GD-401-3001, Illumina) and the cBot instrument (Illumina). Sequencing of 52 bp single reads and seven bases index reads was performed on an Illumina HiSeq 2000 using the TruSeq SBS Kit v3-HS (#FC-401-3002, Illumina). Approximately 20 million reads were sequenced per sample.

For miRNA, the TruSeq Small RNA Library Preparation Kit was used (#RS-200-0012, Illumina) to prepare the cDNA library: As a result of miRNA processing by Dicer, miRNAs contain a free 5’-phosphate and 3’-hydroxal group, which were used to ligate specific adapters prior to first and second strand cDNA synthesis. By PCR, the cDNAs were then amplified and indexed. Using magnetic Agencourt AMPure XP bead-purification (#A63881, Beckman Coulter), large DNAs were separated and small RNAs were enriched. Similar to mRNAs, the samples were finally clustered at 9.6 pM and sequenced, while being spiked into mRNA sequencing samples.
4.4.2 Computational analysis

Illumina sequencing reads were converted to the FASTQ format and aligned to the mouse reference genome (obtained from Ensembl 70) using the STAR program on default settings (277). To increase alignment accuracy, the STAR genome index was generated to include splice junction annotations by applying the options ‘-sjdbGTFfile Homo_sapiens.GRCh37.70.primary_assembly.gtf -sjdbOverhang 49’. Using the Picard tools suite, the SAM output from the STAR aligner was converted to BAM format (http://picard.sourceforge.net). The programs Cufflinks and Cuffdiff version 2.2.1 and the options ‘-u -max-bundle-frags 100000000 - no-effective-length-correction -compatible-hits-norm’ were finally used for gene expression estimation and differential expression analysis (278). Using the RNASe QC program version 1.17, the quality of the sequencing experiments was assessed and verified (279). False discovery rate (FDR)-correction of p-values was calculated using Benjamini-Hochberg multiple testing, and the resulting values are expressed as “q”-values. The log2 of the fold change y/x (fragments per kilobase of exon per million fragments mapped, FKPM) was used as measure for relative gene expression. Unless stated otherwise, genes with a q-value of <0.05 and a log2-fold change of >0.6 or <-0.6 were called “differentially expressed” or “significantly deregulated”. Hierarchical clustering was performed utilizing the Spotfire 6.5.2 software (TIBCO Spotfire, Boston, MA, USA) using complete linkage as method and correlation as distance measure.

Pathway analyses were conducted using the Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA, USA) and the built in functions “Upstream Analysis” as well as “Diseases and Functions” to identify potential upstream regulators and downstream functions likely associated with a set of differentially expressed genes. The number and identity of overlapping genes among two sets of genes was calculated using the IPA function “compare”. EnrichR (http://amp.pharm.mssm.edu/Enrichr) was used to identify enriched “KEGG 2015” and “Reactome 2015” pathways and functions, “PPI hub proteins” and “GO Biological processes”. If necessary, differentially expressed genes were previously filtered using Microsoft Excel 2010 and the data filter function.

For the calculation of correlation and anti-correlation of miRNA and lung function data, all miRNA expression profiles (i.e. from all animals of both, Bleomycin- and AAV-TGFβ1-models) were globally compared to all lung compliance data. This approach was based on the assumption that miRNAs that are directly associated with changes in lung function are highly (anti-)correlated with lung function independent of potential differences in disease course and kinetics. For the prediction of putative mRNAs targeted by a given miRNA, the multiMiR database was used (218).
4.4.3 Statistics

Statistic calculations were performed using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA). Statistical significance between experimental groups was assessed using either t-tests or one- or two-way ANOVA followed by post-tests to correct for multiple comparisons (if appropriate).
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V Record of contributions

I designed, conducted and analyzed all experiments, except from following figures and tasks:

- Intratracheal administrations and lung function measurements in mice were conducted by Helene Lichius or Christopher Tomsic (Respiratory Diseases Research, Boehringer Ingelheim).
- Micro-computed tomography (Figure 16a, Figure 31c) was conducted by the in vivo imaging lab of Dr. Detlef Stiller (Target Discovery Research (TDR), Boehringer Ingelheim).
- Next generation sequencing library preparation and NGS raw data computing were conducted in the computational biology group (TDR, Boehringer Ingelheim) by Werner Rust and Dr. Germán Leparc.
- Ashcroft scoring (Figure 37e) was conducted by Dr. Birgit Stierstorfer, a histopathologist within TDR at Boehringer Ingelheim.
- The analyses in Figure 21, Figure 28 and Table 5 were supported by calculations of Dr. Holger Klein and Dr. Karsten Quast (computational biology group, TDR, Boehringer Ingelheim).
- High-content image analysis calculations (Figure 11c) were performed by Dr. Michael Schuler (TDR, Boehringer Ingelheim).
## VI List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
<td>KEGG</td>
<td>Kyoto encyclopedia of genes and genomes</td>
</tr>
<tr>
<td>AdV</td>
<td>Adenovirus</td>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>AEC</td>
<td>Alveolar epithelial cell</td>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>b.i.d.</td>
<td>Twice daily (lat. <em>bis in die</em>)</td>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
<td>MWCO</td>
<td>Molecular weight cutoff</td>
</tr>
<tr>
<td>Bis-Tris</td>
<td>Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane</td>
<td>o.d.</td>
<td>Once daily</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
<td>poly(A)</td>
<td>Poly-adenylation</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
<td>sc</td>
<td>Self-complementary</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>FMT</td>
<td>Fibroblast-to-myofibroblast transition</td>
<td>ss</td>
<td>Single-stranded</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
<td>TBST</td>
<td>Tris-buffered saline and Tween 20</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
<td>TGFβ1</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
<td>vg</td>
<td>Viral genome</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
<td>wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
<td>x g</td>
<td>Number of times the gravitational force</td>
</tr>
<tr>
<td>hGH</td>
<td>Human growth hormone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRPO</td>
<td>Horse radish peroxidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.t.</td>
<td>Intratracheal</td>
<td></td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
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<tr>
<td>ITR</td>
<td>Inverted terminal repeat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
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