Regenerative Pharmacology for COPD
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Preface

The primary objective of this thesis was to uncover the mechanisms underlying cigarette smoke and air pollution-induced dysfunctional alveolar repair. The second objective was to discover novel therapeutic targets that could be beneficial to promote lung repair in COPD. For this purpose, we established cigarette smoke (CS) as well as Diesel exhaust particles (DEP)-induced experimental models to mimic specific exposures relevant to COPD. We used PCLS as well as lung organoid models to identify the cell-cell interactions, to identify the role of different molecular signaling pathways associated with lung repair and to screen potential drugs for COPD treatment.
Tobacco smoke and diesel exhaust particles lead to dysfunctional responses in alveolar epithelial progenitors

Chronic pulmonary obstructive disease (COPD) has been widely accepted as a condition caused by exposure to tobacco smoke and environmental pollutants. This concept is due to the fact that smoking elicits an abnormal inflammatory response that damages the airways (bronchitis-bronchiolitis) and alveoli (emphysema), accelerates the physiologic decline of lung function with age, and leads to airflow limitation and chronic respiratory symptoms, which are difficult to reverse and may periodically be manifested as exacerbations. However, there is a large proportion of the COPD patient population who are nonsmokers, and other environmental pollutants, such as air pollution, are reported as major environmental risk factors for COPD globally. In this thesis, we mainly focused on one feature of COPD, emphysema, the destruction of alveolar airspaces, which is due to the imbalance between protease and antiprotease activity, enhanced apoptosis, oxidative stress, autoimmunity, malnutrition, or a combination of these factors. Alveolar epithelial progenitors have been elaborated on chapter 1 as being important cells within the alveolar niche regulating alveolar repair. Thus, the biology of alveolar epithelial progenitors is broadly investigated in this thesis, as the main players in the lung repair orchestra.

It is not difficult to imagine that if the lung is constantly exposed to smoking, this will easily cause repetitive inhalational injury to lung tissue, and one puff of cigarette smoke contains millions of toxic chemicals mixtures that will travel through from the upper airway until the distal respiratory system (proximal-distal direction) and consequently settle down in all these parts of the lung due to the different particles sizes. Besides nicotine, thousands of other toxic chemical substances derived from the raw tobacco and added flavors are contained in tobacco smoke. However, unlike tobacco smoke, air pollution results from an even more complex mixture of thousands of pollutants including solid and liquid particles suspended in the air from resources such as energy heating, industrial emissions, and traffic. Thus, the complex compositions of tobacco smoke and air pollution increase the challenge to unravel the specific injury mechanisms and to discover specific drug targets for COPD. As the pathological alterations resulted from smoking or air pollution are not from a single substance/stimulus, we utilized research cigarette smoke (CS) extract as well as diesel exhaust particles (DEP) as disease models respectively in this thesis to investigate the mechanisms of defective lung function.
The exposure time of these stimuli to the lung is essential in determining the extents of injuries. In chapter 5, we established an in vitro organoid model with a constant CS exposure for 14 days, whereas we used an in vivo experimental mouse model for a week of CS exposure. Significant inhibition of organoid formation was observed after both the in vitro and in vivo exposure to cigarette smoke, suggesting the exposure times we selected are sufficient to impact alveolar epithelial progenitors. We also observed a significant impact of DEP in the lung organoid model in vitro in chapter 6, thus it would be of great interest to develop an in vivo model in the future that may provide an additional platform to screen drug targets.

Most experimentally studied mechanisms in COPD induced by either CS or DEP have been linked to inflammation and oxidative stress. In this thesis, we identified different mechanisms using transcriptome analysis. To unravel the mechanisms by which CS (chapter 5) affects alveolar epithelial biology, we performed RNA sequencing on epithelial (CD31-/CD45-/EpCAM+) cells isolated from mice exposed to air or CS. The most significantly enriched molecular pathway from differentially expressed genes upregulated in response to CS is cell cycle, whereas circadian clock, growth factors involved regulations and cell-cell communication were shown downregulated. To uncover the mechanisms by which DEP (chapter 6) affects alveolar epithelial biology, we performed RNA sequencing using re-sorted epithelial cell and fibroblast populations derived from control and DEP-exposed organoids. The top enriched pathway from differentially expressed genes downregulated in response to DEP exposure in both epithelial and fibroblasts populations is extracellular matrix (ECM) organization and the cell cycle pathway is exclusively overrepresented in fibroblast population. Taken together, cigarette smoke and diesel exhaust particles both result in reduced alveolar progenitor cell function but with the involvement of quite different mechanisms.

**Novel therapeutic targets for lung repair**

*Imbalance of canonical and non-canonical WNT signaling*

The maintenance of stem/progenitor cell function is required for lung tissue homeostasis during normal turnover and repair. WNT signaling is implicated in both maintenance as well as the differentiation of adult stem/progenitors. Aberrant expression of the non-canonical ligands WNT-5A and WNT-5B has been demonstrated in lung diseases previously, however, their impact on alveolar
epithelial repair remains largely unknown. In chapter 3, we demonstrated that WNT-5A and WNT-5B, whose expression is enhanced in old murine lungs and in COPD have divergent repressive effects on alveolar epithelial progenitors, whereas the canonical WNT/β-catenin was, however, reduced by both WNT-5A and WNT-5B signaling. Notably, a single WNT ligand can activate multiple signaling pathways depending on the cell-surface receptors present on the cell.\textsuperscript{23,24} Accumulating evidence indicates that WNT-5A binds to receptors such as ROR2 and FZD2, FZD7, FZD8\textsuperscript{25–28}, thereby activating downstream targets involved in regulation of different biological conditions. Thus, it would be interesting to further identify which receptors bind to WNT-5A/5B ligand resulting in the above-mentioned findings, as they could represent drug targets. Notably, pretreatment of fibroblasts with WNT-5A/5B before co-culture with epithelial cells in organoids was not able to repress alveolar epithelial cells growth, suggesting epithelial cells and not fibroblasts are the target cells for WNT-5A/B for this effect. It is of great interest to further explore the WNT dynamics contributing to lung repair to understand how the imbalance of canonical and non-canonical WNT signaling pathways in combination with CS or DEP exposure contributes to defective lung repair. Targeting this imbalance may provide potential drug targets and cell therapies beneficial to COPD treatments.

**Pharmacological ROCK inhibition**

Rho-associated coiled-coil containing kinase (ROCK) is the downstream target of Rho, and two isoforms, ROCK1 and ROCK2 are highly homologous and are widely expressed in the lung.\textsuperscript{29–31} The contribution of the Rho/ROCK signaling pathway in the pathogenesis of pulmonary diseases has been a topic of intense study in recent decades with an increasing interest of using Rho kinase selective inhibitors in studies.\textsuperscript{29–35} However, most published studies focus on the classic ROCK inhibitors, such as Y27632\textsuperscript{35–37} and (hydroxy) fasudil\textsuperscript{29,31,32,34,36,38,39}, both of which target the ATP-dependent kinase domain of ROCK1 and ROCK2 and do not provide good enough selectivity over several other kinases which are also inhibited. In chapter 4, two novel ROCK inhibitors, compound A11 (a ROCK2 selective inhibitor), and compound 31 (a dual ROCK1 and ROCK2 inhibitor) with superior ROCK selectivity, were studied in our PCLS, lung organoid as well as fibroblast models with respect to their therapeutic effects on lung repair. Our findings reveal that pharmacological inhibition of both ROCK 1 and 2 isoforms effectively prevents TGF-β-induced fibroblast myofibroblast differentiation and counteracts TGF-β induced growth inhibition of alveolar epithelial progenitors. Moreover, the selective ROCK2 inhibition does not affect TGF-β effects on extracellular matrix and contractile proteins but does reverse the TGF-β induced
inhibition of organoid growth. Hence, to target mesenchymal ROCK1/2 inhibition may be a potential therapeutic approach to promote lung repair.

According to a recent study, ROCK inhibition using Y27632 may inhibit inflammatory responses and maintain epithelial cell barrier function in response to industrial PM$_{2.5}$ (particulate matter)\textsuperscript{37}. In addition, another study\textsuperscript{37} demonstrated that RhoA/ROCK signaling regulates the airway epithelial inflammation in response to cigarette smoke extract. Taken together, it is rational to explore the role of RhoA/ROCK signaling in combination with our CS and DEP experimental systems in the future with respect to their effects on lung repair.

**Prostaglandin E2 (PGE2) and Prostaglandin I2 (PGI2)**

In chapter 5, we generated a transcriptomics-guided drug target discovery strategy and came up with two fascinating therapeutic potential drug targets, PGE2 and PGI2. Prostaglandins (PGs) are lipid mediators synthesized from arachidonic acid (AA). Among all PGs, PGE2 is a predominant product and exerts diverse biological effects by binding to specific G-protein-coupled receptors (EP1-EP4)\textsuperscript{40,41}. The second messenger molecule cyclic 3',5'-adenosine monophosphate (cAMP) has antifibrotic properties, and PGE2 can generate cAMP production through EP2 and EP4 receptors\textsuperscript{42}, whereas EP3 activation results in decreased cAMP synthesis in cells\textsuperscript{43}, and EP1 activation is coupled to Gq activation and Ca$^{2+}$ signaling particularly\textsuperscript{41}. PGE2 is also a well-known inflammatory mediator as well as an important regulator in tissue repair and injury\textsuperscript{44}. Additionally, PGE2 protected against lung fibrosis before bleomycin challenge\textsuperscript{44}. Prostaglandin I2 (PGI2) is another important lipid mediator of PGs, which are generated by a stepwise conversion of AA as well. PGI2 exerts its biological effects through G-protein-coupled receptor (IP), thereby activating cAMP signaling\textsuperscript{44,45}. Iloprost, a stable PGI2 analog, has been used for asthma, fibrosis and is clinically used for the treatment of atherosclerosis and pulmonary hypertension with regards to its powerful vasodilator action and anti-platelet aggregation\textsuperscript{46–48}.

Although little is known about how PGE2 and PGI2 function in COPD pathogenesis, for the first time we revealed (chapter 5) their beneficial effects in protecting the alveolar epithelial progenitor against the damage resulting from CS exposure both in vitro and in vivo. Subsequently, to clarify the mechanisms involved in these beneficial effects of PGE2/PGI2, we performed RNA sequencing on epithelial (CD31$^{-}$/CD45$^{-}$/EpCAM$^{+}$) cells isolated from mice exposed with CS as vehicle control and CS + misoprostol (PGE2), or CS + iloprost (PGI2). These studies identified the circadian
clock and the cell cycle signaling as the most significantly altered molecular signaling pathways.

Cell cycle and circadian clock signaling

Cell division (cell cycle) is a fundamental cellular process, which is dependent on the activation and de-activation of cyclin-dependent kinases (CDKs) and the oscillatory expression of cyclin proteins, which regulate CDK activities in all cells. Proliferative cells progress through the cell cycle in a process whose completion takes about a day and transition of the proliferating cell from one cell cycle phase to another is tightly regulated by cell cycle checkpoints. In the process of cell growth and division, the cell sustains a sequence of observable changes culminating in mitosis, which is a process that is then reset by daughter cells. The growth and survival of cells as well as the integrity of the genome are regulated by a complex network of pathways, in which cell cycle checkpoints, DNA repair and programmed cell death have critical roles, thus, dysregulation of cell cycle and cell death result in pathological conditions. In both chapter 5 and chapter 6, we revealed cell cycle regulation is a key event underlying the alveolar epithelial dysfunctions resulted from CS exposure as well as DEP exposure. Thus, to further study the checkpoints of alveolar epithelial progenitors undergoing repair in response to CS/DEP may be of great value in the future.

Mammals optimize their physiology to the light-dark cycle by synchronization of the master circadian clock in the brain with peripheral clocks in the rest of the tissues of body. The molecular oscillator of the mammalian circadian clock consists in a dynamic network of genes and proteins and the core mechanism is CLOCK : BMAL1 protein complex the promotes transcription of Period (Per) and Cryptochrome (Cry) mRNA. Moreover, COPD symptoms strongly vary from day to night, being worse in the night and early morning, suggesting the circadian rhythm in COPD patients may be impaired. However, the role of circadian clock in lung repair is unknown.

Both cell cycle and circadian clock are essential for cellular health in mammals, and the circadian clock plays a vital role in regulating the cell cycle, thus affecting cellular proliferation. Interestingly, the cell cycle and the circadian clock represent major cellular rhythms, which appear to be coupled in recent studies. Of note, in chapter 5, using transcriptome analysis, we show that CS induces a wide range of transcriptional effects, including alteration of circadian clock pathway as well as cell cycle which is corrected by either misoprostol or iloprost treatments. However,
whether these two signaling pathways work alone or in cooperation in lung repair are unknown and it would be of great interest to investigate further.

Antioxidants NAC and MitoQ
As oxidative stress is broadly seen in COPD pathogenesis, targeting the imbalance between oxidants and antioxidants is likely beneficial for COPD outcomes. N-acetylcysteine (NAC), a cysteine-donating reducing agent, which when pharmacologically administrated, may reduce cystine moieties to cysteine resulting in GSH elevation. The maintenance of a healthy and functional mitochondria network is critical during both developmental and stressed conditions. Mitochondria are not only the main cellular source of ROS, but they are also susceptible to oxidative injuries. Mitoquinone (MitoQ) is a mitochondrion-targeted antioxidant that was widely used to suppress mitochondrial ROS, suggesting a protective role against oxidative damage-related pathologies in metabolism. Furthermore, mitochondria dysfunction in COPD patients is reported to be associated with excessive mitochondrial ROS production leading to enhanced inflammation as well as cell hyperproliferation. Thus, targeting mitochondrial ROS represents a promising therapeutic approach in COPD. In chapter 6, we examined the efficacies of both NAC and MitoQ on an adult lung organoid model and revealed that these antioxidants restore the repressed organoid growth resulted from DEP exposure. However, whether these rescuing effects are mediated by redox signaling is unclear and to test ROS levels may be a next step to moving forward in the future.

COPD modeling using precision-cut lung slices (PCLS) and lung organoids
Tissue-origin ex vivo and explant cultures, such as precision-cut lung slices (PCLS), and lung organoids are used frequently nowadays for modeling respiratory pathology. PCLS are thin slices of lung tissue that maintain the complex microarchitecture, cellular diversity, and functional response to stimuli observed in native lung tissue, enabling ex vivo mechanistic studies of pathogenesis. In chapter 2, we elaborated the methodology of PCLS in mice together with its application. In chapter 3 and chapter 4, PCLS was used respectively as an essential model to determine the expression of WNT-5A/5B ligands as well as remodeling resulted from TGF-β. However, PCLS can only be cultured for a short duration (around 96
h in our system) *ex vivo*, and the decreased viability and functionality will limit the applications of this model.

Organoids\textsuperscript{10,55,71–75} are an ideal system that provide a platform to study the response of cellular populations to various conditions rapidly and easily where complex *in vivo* analysis is less feasible\textsuperscript{76} and typical 2D cell culture cannot recapitulate the cell-cell interactions. In this thesis, unlike other organoid models only using a single type of cells, our organoid system is derived from freshly isolated epithelial cells (CD31-/CD45-/EpCAM+) co-cultured with fibroblasts. This is because mesenchymal fibroblasts are playing essential roles in supporting biological processes of alveolar epithelial cells, which is also the case *in vivo*. Furthermore, as organoid is a fascinating tool for drug screening, the influence of a drug can be tested in both cell types within one model. Pretreatments on fibroblasts are feasible before inducing to the organoid cultures, thus, in *chapter 3*, the organoid experiment using the fibroblasts pretreated with WNT-5A/5B revealed no impact on organoid formation, however, in *chapter 4*, the pretreatment of TGF-\(\beta\) induced myofibroblasts differentiation to the organoid model helped to identify the effects of ROCK inhibitions subsequently.

In *chapter 3*, we used wild type mice as well as TCF/lef: H2B-GFP mice to isolate epithelial cells for organoid cultures. These WNT-responsive organoids are a powerful tool for investigating the interactions between canonical and non-canonical WNT signaling in lung progenitors. Moreover, an adult organoid model exposed to either CS or DEP was established in *chapter 5* and *chapter 6*, which revealed different molecular signaling pathways are regulating each cell population in response to these stimuli. Therefore, the lung organoid model also provides possibilities to study the crosstalk between alveolar epithelial progenitors and fibroblasts, which opens up new avenues for drug target discovery in lung repair.
Conclusions

In conclusion, the current studies described in this thesis have revealed that cigarette smoke, air pollution, non-canonical WNT signaling and TGF-β all lead to dysfunctional responses in lung epithelial progenitors, which can be reversed pharmacologically (Fig.1). The novel therapeutic targets revealed in this thesis are:

- Mesenchymal WNT-5A/5B result in the inhibition of WNT/β-catenin signaling, thereby repressing alveolar epithelial progenitors. Targeting the imbalance of canonical and non-canonical WNT signaling in the lung may help restore alveolar repair in the ageing lung and in COPD (chapter 3).
- Dual pharmacological inhibition of ROCK1 and 2 isoforms effectively prevented TGF-β induced myofibroblast differentiation and counteracts TGF-β induced epithelial progenitor dysfunction, which indicate that mesenchymal ROCK1/2 inhibition may be a potential therapeutic target to promote lung repair (chapter 4).
- PGE2 and PGI2 had strong therapeutic potentials in correcting the response to cigarette smoke (extract) which might be explained by cell cycle and clock circadian signaling coupling (chapter 5).
- Diesel exhaust particles (DEP) functionally inhibit lung organoid formation by epithelial progenitors, whereas NAC and MitoQ protect against the damage from DEP (chapter 6).

Collectively, these findings provide several promising therapeutic approaches/targets for respiratory diseases associated with defective lung repair.
Figure 1. Schematic summary of the thesis. (A), PGE2, PGI2, NAC and MitoQ are novel drug targets discovered in this thesis in response to either cigarette smoke or diesel exhaust particles induced alveolar dysfunction. (B), Dynamic molecular signaling pathways identified in this thesis regulating the crosstalk between alveolar epithelial progenitor and fibroblast.
References


