Early life exposure to toxic environments: effects on lung and immune cell development in mice and men
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Chapter 4

Interaction of xenobiotic, immune regulatory and DNA repair genes with lung pathology and repair in a (maternal) smoke model

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Abstract: Chronic Obstructive Pulmonary Disease (COPD) is an inflammatory disorder of which the prevalence is particularly high in the elderly population. Several studies suggested that parental smoking may synergize with personal smoking to increase the risk of COPD. We have recently shown that maternal smoking during pregnancy increased airway remodeling and house dust mite-induced goblet cell metaplasia in an experimental mouse model for allergic asthma. In this study, we were interested whether offspring from mothers that were exposed to cigarette smoke during pregnancy were more susceptible to smoke-induced inflammation and tissue remodeling in the lung. This was linked to expression of genes that are important in inflammation, xenobiotic metabolism, anti-oxidant defense, aging and senescence and genes that are related to tissue repair. C57BL/6 mice were exposed to fresh air or cigarette smoke from 1 week prior to conception until birth. When 8 weeks old, offspring was subsequently exposed to cigarette smoke or air for 12 weeks. In lung tissue, inflammation and airway remodeling were investigated by immunohistochemistry, whereas gene expression was assessed by quantitative PCR. We found that maternal smoking during pregnancy down-regulated expression of the anti-inflammatory transcription factor Aryl hydrocarbon receptor (Ahr) in the offspring. In addition, both the anti-oxidant gene Forkhead box class O 3a (Foxo3) and the anti-aging gene Sirtuin1 (Sirt1) were down-regulated in prenatally smoke-exposed offspring. In contrast, expression of airway basal cell-related genes Cytokeratin 5 (Krt5) and Trp63 (P63) were higher in prenatally smoke-exposed mice. Offspring exposed to cigarette smoke for 12 weeks had more inflammation (M2 macrophage infiltration), remodeling (thickening alpha smooth muscle layer), and higher expression of the mucus-related gene Muc5ac, cytochrome P450 family 1 subfamily A member 1 (Cyp1a1) and Ahrr, the repressor of Ahr, irrespective of prenatal smoke exposure. We conclude that prenatal smoke exposure affects gene expression from various pathways related to oxidative stress, lung senescence and repair. Offspring smoking promoted lung inflammation, tissue remodeling and repair, which was not further enhanced by prenatal smoke exposure.
Effect of prenatal and postnatal smoke exposure on lung pathology

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a life-threatening, chronic, progressive lung disease of which the prevalence is particularly high in the elderly population. Patients with COPD are characterized by persistent irreversible airway obstruction and although cigarette smoking is the main cause of development of COPD, other inhaled noxious particles and gases may contribute (1). Different clinical phenotypes of COPD have been described which are associated with prognosis and response to currently available therapies (2). These different phenotypes can be explained by the large variation in lung pathology found in COPD, related to inflammation, cellular apoptosis, extracellular matrix destruction, oxidative stress and abnormal cell repair (3).

For a long time, COPD has been considered to be an adult-onset disease. However, recent studies suggest that COPD may have an early origin and may be initiated even before childhood (4-7). Factors that are associated with increased risk of COPD include delayed lung growth during childhood and adolescence (8-10), childhood exposure to environmental pollutants (11, 12), childhood pneumonia (13) and childhood asthma (14, 15). However, most likely, in particular the interaction between these various risk factors will be important in predisposing to COPD.

Epigenetic mechanisms, including DNA methylation, may be important processes in early-life programming. Interestingly, in human birth cohort studies, maternal smoking during pregnancy was associated with Epigenome-wide DNA methylation differences in (cord) blood cells, fetal lung and placenta, using the Infinium HumanMethylation450k Beadchip (16-22). In these studies, altered methylation of CpG sites could be mapped to genes, amongst others, from the Aryl hydrocarbon receptor (Ahr) pathway that are implicated in the xenobiotics metabolism, the oxidative stress response and immune cell regulation (i.e. Aryl hydrocarbon receptor repressor (Ahrr) and cytochrome P450 family 1 subfamily A member 1 (Cyp1a1)). Differentially methylated CpG sites were confirmed in the different birth cohort studies, but no functional data are available on whether the observed changes in DNA methylation have led to differences in gene or
protein expression (23).

Previous mouse studies from our group have shown that maternal smoking during pregnancy down-regulated Wnt pathway-related genes in neonatal offspring (24). The Wnt family of signaling proteins is known to be important in lung development (25). In addition, maternal smoking during pregnancy increased smooth muscle thickening and collagen deposition around the airways in adult offspring (26), structural changes that are also found in COPD (27).

In this study, we were interested whether offspring from mothers that were exposed to cigarette smoke during pregnancy were more susceptible to postnatal smoke-induced inflammation and tissue remodeling in the lung. Several retrospective, but also prospective studies suggested that parental smoking may synergize with personal smoking to increase the risk of COPD (10, 28, 29). To get some overview in our mouse model on different pathways that are related to the pathogenesis in COPD, the presence of lung infiltration and airway remodeling was linked with expression of a (small) variety of genes regulating (1) immune cell differentiation and cigarette smoke detoxification (Ahr, Ahrr, Cyp1a1), (2) the oxidant defense system, aging and senescence (Foxo3, Sirt1), and (3) tissue repair (Krt5, P63). The different pathways are introduced shortly below.

**The Aryl hydrocarbon receptor pathway**

The AHR protein is a ligand activated transcription factor which is abundantly expressed in the cytoplasm of most, if not all, cell types of the lung (30, 31). In the absence of ligand, the AHR is complexed with chaperone proteins, including a dimer of heat shock protein 90 and p23 (32). Upon ligand binding and activation, AHR dissociates from this protein complex, translocates to the nucleus, heterodimerizes with the AHR nucleus translocater (ARNT) and binds to an enhancer sequence (Dioxin responsive element (DRE)) of its target genes, including Cyp1a1 and Ahrr. The AHRR competes with AHR for binding ARNT, resulting in repression of transcription upon binding of AHRR/ARNT complex to DRE (33).
SIRT1, FOXO3 and aging

Sirtuin 1 (SIRT1) is a nuclear nicotinamide adenine dinucleotide (NAD+)-dependent protein/histone deacetylase, which has been emphasized in a continuously expanding number of functions, such as the regulation of metabolism, cellular survival, autophagy, and organismal lifespan (34-36). When SIRT1 is overexpressed, it deacetylates a variety of proteins, including the transcription factor FOXO3. FOXO3 is involved in mediating the expression of multiple genes involved in cellular development, survival, apoptosis, as well as oxidative stress (37, 38). Once FOXO3 is activated and acetylated in the cytoplasm, it translocates to the nucleus. SIRT1 is an important regulator of transcriptional activity of FOXO3. Deacetylation of FOXO3 by SIRT1 could lead to the induction of a subset of antioxidant genes that regulate cellular detoxification, cell cycle arrest and DNA repair and thus support cell survival (34, 36).

Basal cells and endogenous self-renewal and tissue repair

Recent studies have shown the existence of tissue-specific stem cells in multiple adult organs, which have the capacity for long-term self-renewal and the ability to differentiate into other cell lineages (39). Although the detection of proliferative adult stem or progenitor cells that actively take part in repair and regeneration has been challenging, human and mouse studies have identified three subsets of lung epithelial cells with self-renewal and differentiation capacity, including the basal cells, club cells and alveolar type II cells (39, 40). Signaling pathways that have been described to be important in stem cell self-renewal and lung tissue regeneration include Wnt, Notch and EGF/FGF signaling (41, 42). Interestingly, recent lineage tracing studies in an H1N1 mouse model identified a P63+Krt5+ cell population to be important in alveolar regeneration after a sub-lethal influenza viral infection (43), whereas basal cells expressing P63 and Krt5 are normally abundant in the upper airways to repopulate denuded trachea (44-46).

Material and Methods
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Animals

Female and male C57BL/6 mice, age 8-10 weeks, were obtained from Harlan (Horst, The Netherlands). Mice had access to standard food and water ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (permit number 6589B and 6589C).

Cigarette smoke exposure

Mainstream cigarette smoke was generated using a TE-10 smoke exposure system of Teague Enterprises Smoke Exposure System (Woodland, California, USA). Female mice were exposed to fresh air (n=22) or cigarette smoke (n=26) in two sessions of 50 minutes with a 3h interval between both exposures per day in which smoke of 10 cigarettes were generated per session. Mice were exposed from 7 days before mating until the day of delivery. The adaption protocol included exposure to 3 cigarettes per session the first day, 5 cigarettes the second day, 7 cigarettes the third day and 10 cigarettes the fourth day and thereafter. Smoking 10 cigarettes in one session generated total particulate matter counts of at least 200 mg/m3 and a CO level of 250 PPM (max). Kentucky 2R4F research-reference filtered cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, Kentucky) were used. For experimental purposes, female mice were treated with 5 IU pregnant mare's serum gonadotrophin and 5 IU human chorionic gonadotrophin to induce simultaneous cycling. To induce pregnancy, females were housed one to one with males for 5 consecutive nights. Males were not exposed to cigarette smoke. Mating was confirmed by vaginal plug detection. Smoke exposure remained constant during the total pregnancy. Mothers and offspring were not exposed to cigarette smoke during weaning. Offspring (n=46 from non-smoking mothers, n=25 from smoking mothers) were exposed to air (n=34, n=16 males) or smoke (n=37, n=19 males) when they were 8 weeks old. After 12 weeks of smoke or air exposure, 5 days a week, mice were sacrificed. The left lung was partly used for qRT-PCR analyses and another part was snap frozen and kept at −80 °C. From the right lung, three out of four right lung lobes were snap frozen and kept at −80 °C, whereas the smallest lobe lung was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analyses.
Quantitative Real Time PCR (qRT-PCR) analysis in lung tissue

Total RNA was isolated from lung tissue using a RNA isolation trizol kit (Thermo Fisher Scientific, Carlshad, USA). cDNA was reverse transcribed using a Superscript-II Reverse Transcriptase kit (Thermo Fisher Scientific, Carlshad, USA). To measure the expression of Gapdh (Mm99999915_g1), Ahr (Mm00478932_m1), Ahrr (Mm00477443_m1), Cyp1a1 (Mm00487218_m1), Sirt1 (Mm00490758_m1), Foxo3 (Mm01185722_m1), Muc5ac (Mm01276718_m1), Trp-63 (P63, basal cell, Mm00495793_m1), and Keratin 5 (Krt5, basal cell, Mm01305291_g1), on demand Gene Expression Assays were used (Thermo Fisher Scientific, Carlshad, USA). PCR reactions were performed in triplicate in a volume of 10 µL consisting of 2 µL of MilliQ water, 5 µL PCR master mix (Eurogentic, Belgium), 0.5 µL assay mix and 2.5 µL cDNA. Runs were performed by a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). Data were analyzed with LightCycler® 480 SW 1.5 software (Roche) and the Fitpoints method. RNA data were normalized to Gapdh mRNA expression using $2^{-\Delta\Delta C_{t}}$ (Cp means crossing points). Undetectable Cp values (>40) were interpreted as the maximum Cp value (40).

Immunohistochemistry (IHC)

Sections (3 µm) of formalin-fixed and paraffin-embedded lung tissue were stained for Mac3, (macrophages, rat anti-Mac3, BD Biosciences), alpha smooth muscle (SMA, monoclonal mouse anti-α-smooth muscle actin antibody (Progen Biotechnik, Heidelberg, Germany), collagen III (polyclonal goat-anti type-III collagen antibody, SBA, Birmingham, AL, USA), and goblet cells (Periodic Acid Schiff’s (PAS)), as previously described by us (26, 47). SMA presence directly adjacent to the airway epithelium and collagen III presence directly adjacent to the vessels were quantified in the total lung section by morphometric analysis. The surface of positively stained tissue was expressed as mm$^{2}$ per mm airway or vessel in the total lung section.

M2-dominant macrophages were determined by double staining for Mac3 and YM1 (goat anti-mouse eosinophil chemotactic factor (ECF-L), R&D Systems). To visualize Mac3, an immune alkaline phosphatase procedure was used with Fast Blue BB salt (Sigma Aldrich, Zwijndrecht, The Netherlands) as chromogen. YM1 was visualized with 3-amino-9-ethylcarbazole (Sigma Aldrich) as
chromogen. The number of Mac3 single positive and the number of Mac3-positive/YM1-positive cells were counted manually in parenchymal lung tissue at 20× magnification, and numbers were corrected for the total area of lung tissue section as assessed by morphometric analysis using Aperio ImageScope viewing software 11.2.0.780 (Aperio, Vista, CA).

Eosinophils were determined by staining 4 µm cryosections of lung tissue for cyanide resistant endogenous peroxidase activity with diaminobenzidine (Sigma Aldrich). The number of eosinophils (4 random microscopic fields per lung section) was counted manually in a blinded manner, at 8× magnification and averaged. Neutrophils (Gr1, monoclonal rat-anti-GR1 antibody, BD Biosciences) were counted manually in a blinded manner at 20× magnification and numbers were corrected for the area that was counted (6 fields per section) by morphometric analysis using Aperio ImageScope viewing software 11.2.0.780 (Aperio).

SIRT1-positive cells were determined after staining formalin-fixed and paraffin-embedded lung sections with a polyclonal rabbit-anti-mouse SIRT1 (H-300, sc-15404, Santa Cruz). SIRT1-positive cells were counted at 20× magnification and numbers were corrected for the total area of lung tissue section as assessed by morphometric analysis using Aperio ImageScope viewing software 11.2.0.780 (Aperio). Keratin 5 expression was determined by staining formalin-fixed and paraffin-embedded lung sections with a polyclonal rabbit-anti-mouse Keratin 5 antibody (ab52635, Abcam, Cambridge, UK).

Statistical methods

Results obtained from males were shown. Results of qRT-PCR and IHC were expressed as median and range respectively. When residuals were not normally distributed, appropriate log10 or 1/x transformation of the data was performed. The interaction of the effect of prenatal smoke exposure (smoking during pregnancy) and the effect of postnatal smoke exposure (12 weeks smoke exposure offspring) was tested with a multiple linear regression using SPSS Statistics 22 (IBM, Amsterdam, The Netherlands). When no interaction was found, the effect of smoking during pregnancy and the effect of smoking
offspring were assessed separately with linear regression analysis. These are indicated as “Maternal smoke effect” and “Offspring smoke effect”. To assess differences between subgroups, two-sided Mann-Whitney U tests were used as assessed in Prism v5.0 (GraphPad software, San Diego, CA, USA). Correlations between parameters were established using the Spearman nonparametric correlation test. A value of $p<0.05$ was considered significant.

### Results

**Effect of maternal smoking during pregnancy on (smoke-induced) inflammation and remodeling in offspring**

In order to investigate whether offspring from the smoke-exposed mothers were more susceptible to smoke-induced inflammation, the presence of neutrophils, eosinophils, macrophages and M2-dominant macrophages was investigated. We found that the number of M2-dominant macrophages were increased in offspring that were postnatally exposed to smoke for 12 weeks (Fig 1H, $p=0.012$). This effect was independent from maternal smoking during pregnancy, which did not alter the numbers of the different cell types. Figure 2 shows that in addition to cell infiltration, smooth muscle thickening was higher in the postnatally smoke-exposed offspring (Fig 2D, $p=0.023$), an effect that was also independent from maternal smoking during pregnancy. Prenatal smoke-exposed mice had less smooth muscle thickening than offspring born from an air-exposed mother. Muc5ac, a gene involved in mucus production was also higher in the offspring with postnatal smoke exposure (Fig 2F, $p=0.009$), irrespective of prenatal smoke exposure. Club cells and goblet cells both secrete mucus containing the mucin Muc5ac. Goblet cell metaplasia, however, was not induced by (maternal) smoke exposure, as the PAS staining did not show one single positive cell (Fig 2C). Club cells, however, were abundantly present but still need to be quantified. No effects were found for collagen III expression around the vessels (Fig 2E). Next, gene expressions of the Ahr signaling pathway were investigated. We found that maternal smoking during pregnancy decreased Ahr gene expression in offspring (Fig 3A, $p=0.032$), whereas a trend was found for an offspring smoke effect ($p=0.096$, data not shown). Offspring smoking additionally induced Ahrr and
Cyp1a1 gene expression, which was independent of prenatal smoke exposure (Fig 3B and 3C, p=0.047 and p=0.001, respectively).

Figure 1. Infiltration of inflammatory cells in lung tissue from (prenatally) smoke-exposed mice. Representative pictures and scores of (A, E) Neutrophils (GR1), (B, F) Eosinophils (endogenous peroxidase activity), (C, G) Macrophages (Mac3) and (D, H) M2-dominant macrophages (YM1/Mac3). Original magnification 40× (A) and 20× (B-D). Data represent medians of cell numbers (A, B, D) or Mean±SEM (C). NSM: air-exposed mother, SM: smoke-exposed mother. “Offspring smoke effect” was obtained from a linear regression analysis and indicates a difference between both air-exposed groups versus both smoke-exposed groups.
Figure 2. Airway remodeling in (prenatally) smoke-exposed mice. Representative pictures and quantifications of (A, D) Smooth muscle actin (SMA) around the airways, (B, E) Collagen III deposition around blood vessels, (C) PAS negative airway epithelium and (F) Muc5ac mRNA expression after IHC or qRT-PCR analyses in RNA, isolated from lung tissue. Data represent medians of expression. NSM: air-exposed mother, SM: smoke-exposed mother. “Offspring smoke effect” was obtained from a linear regression analysis and indicates a difference between both air-exposed groups versus both smoke-exposed groups.
Figure 3. Expression of AhR signaling pathway related genes in (prenatally) smoke-exposed mice. Expression of (A) Ahr, (B) Ahrr, and (C) Cyp1a1 was analyzed by qRT-PCR in RNA, isolated from lung tissue. Data represent medians of expression. NSM: air-exposed mother, SM: smoke-exposed mother. The “Offspring smoke effect” was obtained from a linear regression analysis and indicates a difference between both air-exposed groups versus both smoke-exposed groups. The “Maternal smoke effect”, obtained from a linear regression analysis, indicates a difference between both NSM-exposed groups versus both SM groups.

Maternal smoking during pregnancy decreases SIRT1 and FOXO3 expression in offspring

We then investigated expression of Sirt1 and Foxo3, genes which are important in the antioxidant defense system and implicated in aging and senescence. Figure 4 showed that the prenatally smoke-exposed mice had a lower mRNA expression of Sirt1 (Fig 4A, p=0.052) and lower numbers of SIRT1-positive cells in lung tissue (Fig 4D, p=0.053). Furthermore, Foxo3 mRNA expression was also lower in prenatally exposed mice (Fig 4B, p=0.053). Expression of Sirt1 was highly correlated with expression of Foxo3 (r²=0.78, p<0.0001, Fig 4C). The postnatally smoke-exposed mice had lower numbers of SIRT1 positive cells than the air-exposed offspring (Fig 4D, p=0.012). IHC staining of SIRT1 indicated that SIRT1 was expressed in the nucleus.
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Figure 4. Lower expression of aging-related markers SIRT1 and FOXO3 in lungs of prenatally smoke-exposed mice. Expression of (A) Sirt1, (B) Foxo3, (C) the correlation plot of Sirt1 expression versus Foxo3, and (D) the numbers of SIRT1 positive cells after qRT-PCR analysis and IHC staining of SIRT1 (Brown, E) in lung tissues of prenatally smoke-exposed mice. Original magnification was 20×. Data represent medians of expression. NSM: air-exposed mother, SM: smoke-exposed mother. The “Maternal smoke effect” was obtained from a linear regression analysis and indicates a difference between both NSM-exposed groups versus both SM groups. p<0.05 and p<0.001 are from post-hoc subgroup analyses by Mann-Whitney U test. Correlation was established using the Spearman nonparametric correlation test.

Maternal smoking during pregnancy promotes basal cell markers in offspring.

To investigate whether repair-related pathways were induced by maternal smoking during pregnancy, expression of the basal cell markers Krt5 and P63 were investigated. As shown in figure 5A, offspring smoke exposure increased
the gene expression of Krt5, an effect that was weaker in offspring from smoking mothers (indicated by a significant negative interaction between the effects of smoking during pregnancy and offspring smoke exposure). In addition, prenatally smoke-exposed mice had a higher mRNA expression of P63 in lung tissue. When staining for Keratin 5 in lung tissue by IHC, a few positive cells could be found in the conducting airway (Fig 5C) and/or alveoli (Fig 5D) in each of the (maternal) smoke-exposed mice. In Figures 5C and 5D, representative photographs of the scarce positive cells that were present in conducting airways and alveoli were shown.

Figure 5. Higher expression of basal cell markers Krt5 and P63 in lungs of (prenatally) smoke-exposed mice. Gene expression of Krt5 (A), P63 (B) and Krt5 expression in a distal conducting airway (C) and alveolus (D) after immunohistochemical staining of Krt5 (brown) in lung tissues of prenatally smoke-exposed mice. Original magnification 20× (C) and 40× (D). NSM: air-exposed mother, SM: smoke-exposed mother.
Discussion

In this study we have found that prenatal smoke exposure down-regulated smooth muscle thickening (SMA) and affected expression of a number of genes, implicated in inflammation (Ahr), the cellular stress response, aging and apoptosis (Foxy3 and Sirt1) as well as tissue repair (Krt5 and P63). In addition, 12 weeks of postnatal smoke exposure induced infiltration of inflammatory cells (M2-dominant macrophages) and promoted airway remodeling (SMA). This was accompanied by a higher gene expression of the mucus-related Muc5ac, xenobiotic-metabolizing enzymes Ahrr and Cyp1a1 as well as the basal cell marker Krt5. There was a negative interaction between maternal smoking and offspring smoking for the expression of Krt5, which means that the effect of offspring smoke exposure was smaller in the prenatal smoke-exposed mice.

(Maternal) smoking and the Aryl hydrocarbon receptor pathway

We found that prenatal smoke-exposed mice had a lower expression of Ahr. This is of interest as Ahr expression has been shown essential for regulating proliferation and preventing mitochondrial dysfunction and apoptotic cell death caused by cigarette smoke (48). In that study, absence of Ahr expression led to the decreased levels of superoxide dismutases MnSOD and CuZNSOD. These are enzymes that protect against oxidative stress (49). This indicates that loss of Ahr expression could contribute to development of COPD. Further analyses of SOD expression, as well as the presence of apoptotic cell death are of interest in our model.

Postnatal smoke-exposed offspring had an increased expression of Ahrr and Cyp1a1. This is of interest as a recent genome wide methylation study in four prospective cohorts showed that smoking-induced expression of Ahrr and hypomethylation in the Ahrr gene were associated with increased risks for subsequent lung cancer (50). In addition, the increased CYP1A1 activity and expression also has been linked recently to development of tobacco-related lung cancer (51). Besides that, numerous studies have implicated a role of P450 enzymes, including CYP1A1, in the formation and further reactions of reactive oxygen species (ROS) (52), contributing to oxidative stress and tissue damage.
However, a protective role for CYP1A1 in tissue damage has also been described. A recent study in a mouse model for oxygen-induced bronchopulmonary dysplasia (BPD) showed that prenatal treatment of pregnant mice with the Cyp1a1 inducer (β-napthoflavone) protected newborns exposed to hyperoxia from lung injury (53). Both Ahrr and Cyp1a1 genes were shown to be differentially methylated in human cord blood studies due to maternal smoking (16-21). In these studies, Ahrr was also consistently found to be hypomethylated, whereas mRNA expression was suggested (trend) to be higher in smoke-exposed cord blood cells (23). We did not find a maternal smoke effect for these genes, probably due to the large variation observed in our groups. However, whether smoke exposure affected DNA methylation in the Ahrr gene will be subject of further studies.

(Maternal) smoking, accelerated aging and SIRT1

Since a few years now, COPD has been characterized by premature aging because of the presence of chronic inflammation (inflammaging) and accelerated decline in lung function (54-56). The process of cellular senescence and aging is characterized by activation of several signaling molecules such as FOXO, Klotho, NF-κB and enhanced levels of proinflammatory cytokines (57). It has been shown that these processes are largely controlled by SIRT1 (58-61). Interestingly, SIRT1 was shown to be reduced in lungs of smokers and patients with COPD (62, 63). In addition, treatment of mice with a selective SIRT1 activator, SRT2172, blocked the increase of matrix metalloproteinase-9 (MMP-9) expression in the lung as well as pulmonary neutrophilia, which are both important in development of emphysema (63). In another mouse model for emphysema, SIRT1 activation either by genetic overexpression or a selective pharmacological activator SRT1720, attenuated stress-induced premature cellular senescence and protected against emphysema induced by cigarette smoke. Of note, for the protection of emphysema, deacetylation of the transcription factor FOXO3 by SIRT1 was required (64). In COPD patients and mouse lungs exposed to cigarette smoke, FOXO3 degradation and acetylation was found to be increased (65).

In our study, prenatally smoke-exposed mice had a reduced expression of both
Sirt1 and Foxo3. Furthermore, postnatal smoke-exposed offspring had lower numbers of SIRT1 positive cells. This is of interest as both Sirt1-deficient and Foxo3-deficient mice have been shown to have an increased susceptibility to develop emphysema (65). In our study, emphysema was not investigated and additional negative effects of prenatal smoke exposure on the offspring smoke effects were not observed for both Sirt1 and Foxo3 expression. However, it could be that 12 weeks of smoke was not long enough and that detrimental effects will only become apparent when mice were exposed for a longer period or when they were exposed at an older age.

(Maternal) smoking, epithelial cell homeostasis and tissue repair by endogenous adult lung stem cells

Nonreversible expiratory airflow limitation and chronic cough in COPD are thought to result from deregulated chronic inflammation, airway remodeling and emphysematous destruction of the lungs. Inflammatory cells that are connected to tissue breakdown in COPD are neutrophils and macrophages, producing ROS, proteinases and MMPs (66, 67). In our model we were interested to investigate whether prenatal or postnatal smoke exposure induced tissue injury that would be small enough to be repaired by endogenous adult stem cells such as Krt5 and P63 positive basal cells that normally line the trachea and large bronchi. We found a higher expression of Krt5 and P63 gene expression in lungs from the prenatally smoke-exposed mice, which suggest an increased presence of basal cell in the lung parenchyma. Furthermore, IHC staining for Keratin 5 did show some keratin 5 positive cells in lungs from offspring exposed to (maternal) smoke, but these numbers were really low. Additional studies on lung regeneration and apoptosis should give us more insight into the effects of (prenatal) smoke exposure in our model.
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Summary

We have found that cigarette smoke exposure during pregnancy had detrimental effects on expression of genes from various signaling pathways that are involved in oxidative stress and cell survival. Additionally, expression of the basal stem cell markers P63 and Krt5 were up regulated and some presence of Krt5 positive cells could be found. Prenatal smoke exposure did not further increase the effect of offspring smoke exposure on lung inflammation, remodeling and relevant gene expression. Additional analyses in the lung tissue from these mice with respect to the inflammatory response, apoptosis, oxidative stress and repair, as well as investigation of epigenetic regulation of the differentially expressed genes are necessary for a more in depth insight into the effect of prenatal smoke exposure on cigarette smoke-induced lung pathology later in life.

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