Lung epithelial cell differentiation in human and mouse

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Lung epithelial cell differentiation in human and mouse:

environment, epigenetics and epigenetic editing

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General introduction

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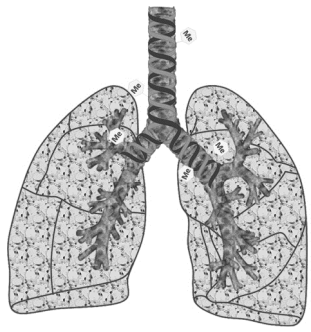

Chronic obstructive pulmonary disease (COPD) is a chronic, progressive inflammatory lung disease characterized by persistent airflow limitation due to mucus hypersecretion (chronic bronchitis) and/or destruction of alveolar walls in the parenchyma (emphysema). Cigarette smoking is the leading cause of COPD. COPD has no cure so far. However, lifestyle changes (e.g. quit smoking) and treatments (e.g. mucus reduction) can improve the patients' quality of life and also slow the progress of the disease. Epigenetics (the heritable, yet reversible, changes in gene expression not encoded in the DNA sequence) might provide new insights to the pathogenesis of COPD and as such might result in the identification of novel therapeutic targets.

Maternal smoking during pregnancy and development of asthma and COPD

Maternal smoking during pregnancy is associated with reduced lung function at birth [1], during childhood [2-4], in adolescence [5] and in adulthood [6-8]. In addition, prenatally exposed individuals have an increased risk for development of wheezing and asthma in childhood [9-11], as well as an increased risk for both asthma and COPD in adulthood [8,12]. Moreover, maternal smoking may synergize with personal smoking to increase airflow limitation and COPD prevalence in adults [13-15], which suggest that maternal smoking during pregnancy might alter early lung development and lifelong affect subsequent lung disease [16]. Several experimental animal studies have shown that maternal smoking during pregnancy affects the immune system (increase of alveolar macrophage numbers and IL-13 levels [17-19]), lung structure (abnormal airway geometry and airway wall composition [20,21]) and lung function (airway hyper-responsiveness and airflow restriction [18,20,21]) in the offspring. However, the mechanisms that underlie these abnormities are largely unknown. Certain genetic variants were shown to be associated with increased risk of asthma and that risk is further increased by early-life smoke exposure[22]. Genetic variants (e.g. IL-13 polymorphism) were shown to modify the impact of prenatal smoke exposure on childhood

asthma [23]. Both smoke exposure (current or prenatal smoke exposure) and genetic variants were shown to be able to impact DNA methylation [24,25] and they may interact to affect DNA methylation[26]. Joint effects of genetic variants and DNA methylation were shown on lung function growth across adolescence [27] and risk of asthma [26,28]. In turn, genotype and methylation of drug metabolizing enzymes, such as CYP2D6, were shown to have an effect on smoking behavior [29]. Altogether, prenatal maternal smoking, genetic variants and epigenetics (DNA methylation) might all contribute to the pathogenesis of complex diseases like asthma and COPD, and epigenetics likely plays the role of integrator of multiple disease pathway signals[26,30].

Epigenetics

Epigenetics refers to molecular mechanisms underlying the epigenetic traits "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence"[31,32]. The term “epigenetics” was originally coined by C. H. Waddington to explain the cell differentiation from a pluripotent stem cell to multiple terminally differentiated cells, having the same genotype but distinct phenotypes [33]. As such, epigenetics is important in the maintenance of gene expression patterns which last through cell divisions for the duration of the cell’s lifespan, and may even last for multiple generations of the organisms [34-36]. Epigenetic mechanisms also play a role in modulating gene expression in response to the environment (such as nutrition and toxins) to which cells are, or the organism is, exposed to. So, epigenetic processes play a key role in gene regulation, and recent studies indicate the relevance of epigenetic dysregulation underlying various disease phenotypes, such as lung disease COPD and asthma [37,38].

In the nucleus, the DNA is wrapped around protein structures, made up by histone proteins. Modifications of these histone proteins as well as DNA methylation of -for example- gene promoters are two extensively

characterized epigenetic mechanisms that regulate gene expression and influence the cellular phenotype without altering the genotype [32,39,40].

DNA methylation is a covalent modification that occurs predominantly at cytosine residues in cytosine-phosphate-guanine (CpG) dinucleotides in mammalian cells [41,42]. DNA methylation is catalyzed by DNA methyltransferase enzymes (DNMTs), which are responsible for transferring a methyl group to DNA, using S-adenosyl methionine (SAM) as the methyl donor. Three active DNMTs have been identified in mammals, DNMT1, DNMT3A, and DNMT3B [43]. DNMT1 preferentially methylates hemimethylated DNA, which contributes to the heritability of DNA methylation during cell division and differentiation. DNMT3A and DNMT3B can methylate both hemimethylated and unmethylated CpGs, and are required for the initiation of *de novo* methylation *in vivo*. In general, DNA methylation of the promoter and first exon is an important mechanism for transcription silencing, either by directly interfering with the binding of proteins necessary for transcription, or by indirectly engaging the recruitment of methyl-binding proteins and co-repressors [40,44,45].

Histone modification is a covalent posttranslational modification that occurs at certain amino acids of the histone proteins, such as methylation or acetylation of lysine and arginine, and phosphorylation of serine or threonine. As the DNA strand is wrapped around the histone proteins to form the nucleosome unit, histone modification can alter their interaction with DNA and nonhistone regulatory proteins, and further change the chromatin accessibility and transcriptional activity. There are five important histone modification marks characterizing distinct chromatin states [40]: histone H3 lysine 4 trimethylation (H3K4me3), associated with promoter regions; H3 lysine 4 monomethylation (H3K4me1), mainly associated with enhancer regions; H3 lysine 36 trimethylation (H3K36me3), associated with transcribed regions; H3 lysine 27 trimethylation (H3K27me3), associated with Polycomb repression; and H3 lysine 9 trimethylation (H3K9me3), associated with repression of, for example, repetitive regions. Two additional histone marks: acetylation marks H3K27ac and H3K9ac, are

associated with increased activation of enhancer and promoter regions. The histone modifications are dynamically deposited or removed by a group of well characterized enzymes, which also respond to environmental or developmental signals. To some extent, the histone modifications are also heritable. For example, evidence in fission yeast, worm and human showed that H3K9me and H3K27me transmitted a memory of repression across generations through meiosis and during development through mitotic divisions [46-49]. Recently, epidemiological and experimental studies showed evidence for inter- and transgenerational inheritance of a smoke (nicotine) effect on increased risk of childhood asthma, which may last for two-three generations [34,50-52].

Airway epithelial cell differentiation, transcriptional profiles and DNA methylation

Conducting airways of the human lung, equivalent to the trachea of mice, are lined with a pseudostratified epithelium containing primarily of basal cells, Club (Clara) cells, ciliated cells and goblet cells [53,54]. Airway basal cells are a population of multipotent stem cells that have been shown to drive both the normal epithelium renewal and abnormal remodeling after injury [55-57]. Selective expression of transcription factors dictate proper epithelial cell differentiation (Figure 1) [58], including Foxj1 (ciliated cell differentiation [59]), Foxm1, Spdef, Foxa2 and Nkx2-1 (goblet cell differentiation [60-63]), Trp-63 and Keratin 5 (basal cell markers, [64,65]). Goblet cell metaplasia, a common feature of chronic obstructive pulmonary disease (COPD) is associated with mucus hyperproduction which contributes to the mortality and morbidity of patients [66-68]. Targeting mucus hypersecretion is hypothesized as a therapeutic target for COPD [69,70].

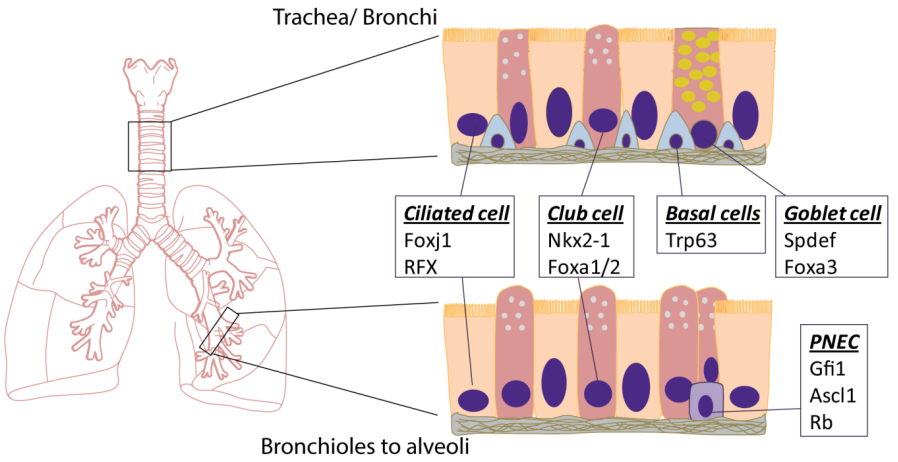

Figure 1 Expression of transcription factors that influence cell differentiation in the airway epithelium. *In the mouse, large conducting airways (trachea and bronchi) are lined by a pseudostratified epithelium consisting of ciliated, Club, goblet and basal cells. Smaller peripheral conducting airways are lined by a simple columnar epithelium containing primarily ciliated and Club cells. Pulmonary neuroendocrine cells (PNEC) are a relatively rare cell type.*

Primary airway epithelial cells isolated from human bronchial or mouse trachea tissue are able to grow on porous supports at an air–liquid interface (ALI) and differentiate into pseudostratified mucociliary epithelium, which is a relevant *in vitro* (normal or disease) model of airway epithelial cell differentiation[71-74]. Goblet cell differentiation was shown to be sufficiently induced in a conditional transgenic mouse model after targeted pulmonary expression of Interleukin (IL)-13 using the Clara cell 10-kDa protein promoter [75], and can be induced *in vitro* after air–liquid interface (ALI) cell culture [76,77].

DNA methylation is an important mechanism for the regulation of gene expression during adult stem cell renewal and differentiation, as shown in hematopoietic, epidermal, and intestinal stem cells [78-81]. However, the function of DNA methylation in the airway basal cell differentiation has not been

evaluated. DNA methylation was shown to be globally disrupted in small airways epithelial samples obtained from bronchoscopic brushings in both healthy smokers and COPD smokers [82,83]. In addition, basal cells from small airway epithelium of healthy smokers and COPD smokers were limited in their ability to regenerate a fully differentiated epithelium ex vivo, which was associated with an altered DNA methylation profile [84].

Targeting DNA and Histones: Epigenetic Editing

Epigenetic editing refers to targeted rewriting of epigenetic marks to modulate expression of selected target genes by using epigenetic editors which comprise engineered gene-specific DNA binding domains and catalytic domains from an epigenetic enzyme [85]. Recently developed DNA targeting systems include zinc fingers (ZFs), transcription activator-like effectors (TALEs) and the clustered regularly interspaced short palindromic repeats (CRISPRs)-dCas9 complex (where the nuclease activity of Cas is mutated to result in a dead Cas9 (dCas)). All of these DNA binding domains can be custom engineered to target any genomic loci. Chromatin-modifying enzyme domains include writers and erasers of DNA methylation and histone modifications. Epigenome editing has been shown to be a powerful approach for functional studies of locus-specific chromatin modification and gene expression, and might also provide a therapeutic approach in the clinic for durable regulation of disease-related genes (e.g. reactivating silenced tumor suppressors or repressing oncogenes [86,87]) and in cellular reprogramming, such as application in reprogramming the differentiated somatic cells to pluripotent stem cells instead of genome editing [88]. The CRISPR-dCas9 system is particularly suitable to high-throughput screens because its targeting is based on a cheap and flexible system where a single guide RNA pairs with one strand of its target DNA. ZFs have the advantage that these proteins are of human origin and may demonstrate less immunogenicity in clinical application than TALE and CRISPR/Cas9 system (of bacterial origin). However, specificity is an important issue for the application in humans and therefore, in this case, TALE and CRISPR/ Cas9

system may be a better choice because of the higher specificity [89-92]. Finally, the delivery strategy of the targeted Epigenetic Effectors is important to address in future studies. In particular, nanoparticles may be a promising alternative of the currently used viral delivery system, and tissue/ cell specificity need to be improved.

Targeted gene repression, so far, is commonly established by so called artificial transcription factors (ATFs)[93,94] linking the engineered DNA binding domain to, for example, Kruppel-assocated box (KRAB) domain, a transcriptional repression domain present in approximately 400 human zinc finger protein-based transcription factors (KRAB zinc finger proteins)[95]. However, as the KRAB domain has no catalytic activity and transcriptional repression is indirectly induced by recruiting a heterochromatin-forming complex [96], its repression effect was shown to be transient [97,98]. Epigenetic editing has the potential to be stable and heritable and Stolzenburg et al indeed showed promising stable silencing effect for targeted methylation of both SOX2 and MASPIN [97,98]. In contrast however, Kungulovski et al could not demonstrate stable silencing results for targeted methylation of the VEFG-A gene [99]. So the stability and heritability of epigenetic editing might correlate with the local chromatin context of the particular targeted loci and still need to be further investigated for more target genes.

Scope of this thesis

The aim of this thesis was to investigate the airway epithelial cell differentiation in human and mouse lung in relation to environmental smoke, epigenetic mechanisms and epigenetic editing to reduce mucus production. In **chapter 2**, the effect of prenatal smoke exposure on airway epithelial cell development was examined in neonatal offspring and further the Notch signaling pathway was assessed. In **chapter 3**, the question was addressed whether offspring from mothers that were exposed to cigarette smoke during pregnancy were more susceptible to (smoke-induced) inflammation and tissue remodeling. In **chapter 4**, the expression level and DNA methylation level of SPDEF and FOXA2 was assessed during airway epithelial cell differentiation and a comparison between patients with COPD and controls was made. In **chapter 5**, the aim was to reduce lung epithelial mucus production by targeted silencing of SPDEF using the novel strategy of epigenetic editing. Finally, the main findings of this thesis were summarized and future perspectives were discussed in **Chapter 6**.

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Chapter 2

Smoking during pregnancy inhibits ciliated cell differentiation and up regulates secretory cell related genes in neonatal offspring

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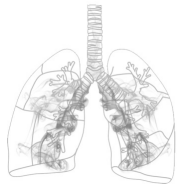
Submitted



Abstract

Maternal smoking during pregnancy is an independent risk factor for children to develop asthma and respiratory infections. We have recently shown that maternal smoking during pregnancy increased house dust mite-induced goblet cell metaplasia in an experimental mouse model. To further study the effects of prenatal smoke exposure on airway epithelial cell development in neonatal offspring, C57Bl/6 mice were exposed to fresh air or cigarette smoke from 3 weeks prior to conception until birth. Offspring was all sacrificed 1 day after birth. In lung tissue, numbers of ciliated cells and secretory Club cells were investigated by immunohistochemistry, as well as mRNA expression of transcription factors that regulate ciliated cell and secretory cell differentiation. We found that maternal smoking inhibited the number of ciliated cells, as well as expression of the major cilia-related transcription factor Forkhead box J1 (*Foxj1*) in offspring. In addition, increased expression of transcription factors involved in secretory cell differentiation, such as Forkhead box M1 (*Foxm1*) and Sam pointed domain-containing ETS transcription factor (*Spdef*) was found in offspring from smoke-exposed mothers. This was accompanied by higher expression of Hey1 suggesting that Notch signaling may be involved in the observed aberrant epithelial cell development after prenatal smoke exposure. The lower number of ciliated cells could affect mucociliary clearance and may explain the increased susceptibility of children from smoking parents to wheeze and to development of childhood respiratory infections.

Keywords: maternal smoking; pregnancy; asthma; ciliated cell; Spdef; Notch

Introduction

The trachea and main bronchi are lined by a pseudostratified epithelium that is composed of ciliated cells, Club cells (formally known as Clara cells) and basal cells (Crystal, et al. 2008; Herriges and Morrisey. 2014) as major cell types . In humans, the basal cells underlie the ciliated and secretory (Club and goblet) cells. A similar epithelial architecture is present in the mouse, although it is limited to the trachea and the largest bronchi. Ciliated and Club cell types act as the front-line defense in a coordinated manner to protect the lungs from inhaled pathogens and noxious agents. Thus Club cells secrete a layer of mucus, trapping inhaled harmful particles and pathogens, whereas ciliated cells clear the latter from the airways by ciliary beating, generating a one way wave-like movement across the epithelial surface (Knowles and Boucher. 2002). Lineage studies in mice and in vitro suggest that most lung epithelial cell lineages have (self-)renewal capacity and (re)generate secretory and ciliated epithelial cell phenotypes both during development and in response to injury (Park, et al. 2006; Sun, et al. 2013; Kotton and Morrisey. 2014). This process is controlled in part by Notch signaling, which promotes secretory cell fate and inhibits ciliary cell differentiation (Guseh, et al. 2009; Rock, et al. 2009; Rock, et al. 2011). In addition, goblet cell metaplasia is dictated by the transcription factors SPDEF, FOXM1, FOXA2 and NKX2-1 (Park, et al. 2007; Maeda, et al. 2011b; Ren, et al. 2013) whereas development of ciliated cells is driven by FOXJ1 (Brekman, et al. 2014). Of note, murine goblet cells are derived from Club cells and arise only after injury or in disease states (Pardo-Saganta, et al. 2013).

In an experimental mouse model for asthma, we have previously shown that smoking during pregnancy substantially increased goblet cell numbers in the airways of 10-weeks old offspring after 5 weeks of house dust mite (HDM) exposure (Blacquiere, et al. 2009). This observation is of interest as it is in line with several birth-cohort studies in which maternal smoking during pregnancy was shown to be a risk factor for the development of transient early wheeze (Civelek, et al. 2011; Caudri, et al. 2013) as well as asthma,

extending into adulthood (Civelek, et al. 2011; Caudri, et al. 2013; Grabenhenrich, et al. 2014). In addition, children born from mothers who smoked during pregnancy have a higher risk to develop respiratory infections (Broughton, et al. 2005).

In the current study, we therefore aimed to get more insight into epithelial cell differentiation and gene expression, directly after birth, in prenatally smoke-exposed mice, to explain enhanced goblet cell differentiation susceptibility later in life. This was addressed firstly by analyzing the number of ciliated cells and Club cells in the conducting airways and secondly by analyzing the expression of genes that dictate proper epithelial cell differentiation.

Material and Methods

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. The animal study was approved by the Institutional Animal Care and Use Committee of the University of Groningen (Permit Number: 6589a) and was performed under strict governmental and international guidelines on animal experimentation.

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=3) or cigarette smoke (n=4) 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 sacrifice. 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/m^3 and a CO level of 250 PPM (max). Kentucky 2R4F research-reference filtered cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, Kentucky, USA) were used. For experimental purposes, female mice were treated with 1.25 IU pregnant mare's serum gonadotrophin and 1.25 IU human chorionic gonadotrophin to induce simultaneous cycling. To induce pregnancy, 1 female was housed with 1 male. Mating was confirmed by vaginal plug detection. Smoke exposure remained constant during the total pregnancy. Mothers and offspring were not exposed to cigarette smoke after offspring was born. Offspring (n=16 from non-smoking mothers, n=24 from smoking mothers) was sacrificed one day after birth. The left lung was used for qRT-PCR analyses. The right lung was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analyses.

qRT-PCR analysis in lung tissue

Total RNA was isolated from lung tissue using a RNA isolation trizol kit (Invitrogen, USA). cDNA was reverse transcribed using a Superscript-II Reverse Transcriptase kit (Invitrogen, USA). To measure the expression of *Gapdh* (assay ID: Mm99999915_g1), *Foxj1* (ciliated cell, Mm01267279_m1), *Scgb1a1* (*Cc10*, Club cell, Mm00442046_m1), *Calca* (neuroendocrine cell, Mm00801463_g1), *Trp63* (*p16*, basal cell, Mm00495793_m1), and Keratin 5 (*krt5*, basal cell, Mm01305291_g1), *Foxm1* (Mm00514924_m1), *Spdef* (Mm00600221_m1) and *Muc5ac* (Mm01276718_m1), *Foxa2* (Mm01976556_s1), *Foxa3* (Mm00484714_m1), *Nkx2-1* (Mm00447558_m1), *Hoxa5* (Mm04213381_s1) and *Hoxb5* (Mm00657672_m1), *Notch1* (Mm00435249_m1), *Notch2* (Mm00803077_m1), *Notch3* (Mm01345646_m1), *Hey1* (Mm00468865_m1), *Hey2* (Mm00469280_m1), *Hes1* (Mm01342805_m1), on demand Gene Expression Assays were used (life technologies, 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 (Eurogentec, Seraing, Belgium), 0.5 µL assay mix (life technologies, USA), 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, Basel, Switzerland) and the Fitpoints method. RNA data were normalized to Gapdh mRNA expression using $2^{-\Delta Cp}$ (Cp means crossing points). Undetectable Cp values of the genes of interest (>40) were interpreted as the maximum Cp value (40).

Immunohistochemistry

Sections (3 µm) of formalin-fixed and paraffin-embedded lung tissue were stained for ciliated- or Club (previously Clara) secretory cells using standard immunohistochemical procedures. Briefly, slides were deparafinized and put in citrate buffer in a microwave oven for 15min. After cooling, slides were incubated in 0.3% H_2O_2 (v/v) in PBS for 30 min. To visualize ciliated cells, slides were incubated with mouse-anti-acetylated a-tubulin at 1:10000 for 1h (Sigma-Aldrich, Zwijndrecht, The Netherlands), which was detected with HRP conjugated rabbit-anti-mouse (1:200, Dako, Glostrup, Denmark) for 0.5h. A 0.05% (w/v) diaminobenzidine (DAB, Sigma-Aldrich, Zwijndrecht, The Netherlands) solution was used for color reaction. To visualize Club secretory cells, a rabbit- anti-Club cell 10 kD (CC10) antibody (1:6000, 1h, Millipore, Billerica, USA) was used as first antibody and a HRP conjugated goat-anti-rabbit antibody (1:200, 0.5h) was used as second antibody. A 0.05% DAB solution was used as chromogen. Numbers of tubulin positive cells and CC10 positive cells were counted manually in all airways. The length of all airways was measured at the basal end of the airway epithelium using Aperio ImageScope viewing software 11.2.0.780 (Aperio, Vista, USA) and the total number of tubulin- and CC10-positive cells was expressed per µm airway.

Double staining of tubulin and CC10 was performed. Briefly, the slides were incubated with a rabbit- anti-CC10 antibody (1:6000, 1h), a goat anti-rabbit

biotin-conjugated antibody (1:200, 30 min) and streptavidin-alkaline phosphatase (1:200, 30 min). After wash steps, slides were incubated in fast blue solution for 30 min to visualize color. The slides were subsequently incubated with mouse-anti-acetylated a-tubulin (1:10000, 1h) and HRP-conjugated rabbit-anti-mouse antibody (1:200, 0.5h) after wash steps, and then incubated in a 0.05% DAB staining solution for 10 min.

Statistical methods

Results obtained from qRT-PCR and IHC are expressed as median and range respectively. The effect of maternal smoking during pregnancy was investigated with a multiple linear regression using SPSS Statistics 22 (IBM, Amsterdam, The Netherlands). When residuals were not normally distributed, appropriate log10 or 1/x transformation of the data was performed. The interaction of the effect of smoking during pregnancy and the effect of sex was tested and not present, implying that the effect of prenatal smoking was similar in females and males. A value of $P<0.05$ was considered significant.

Results

Maternal smoking during pregnancy inhibited ciliated cell differentiation in offspring.

In order to investigate whether the previously observed HDM-induced goblet cell susceptibility in offspring from smoke-exposed mothers was due to the presence of more Club cells in prenatally exposed neonates, we first investigated the mRNA expression of *Scgb1a1*, a marker for Club cells. Figure 1a shows no difference in gene expression of *Scgb1a1*, which was supported by no difference in Club cell numbers in both offspring groups, as analyzed by immunohistochemistry (Figure 2a-c). We then moved on to investigate gene expression of the ciliated cells (*Foxj1*) and neuroendocrine cells (*Calca*) as well. As shown in figure 1b, offspring from smoke-exposed mothers had lower expression of *Foxj1* ($p< 0.05$) than offspring from air-

exposed mothers. Quantification of ciliated cells confirmed that numbers of ciliated cells were indeed lower in offspring from smoke-exposed mothers (Figure 2d-f, p< 0.01). Gene expression of *Calca* (Figure 1c) was not different in both groups of offspring. As, in mouse, the Club cell is thought to be the progenitor of the goblet cell, and as the ciliated cells and Club cells are the two major cell types in the mouse proximal airways, increased numbers of Club cells in offspring from smoke-exposed mothers were expected. Therefore, in a next experiment, the presence of Club cells and ciliated cells was visualized in a double staining for CC10 (Club cells) and tubulin (ciliated cells) and confirmed that both stainings did not overlap, as shown in figure 3. To investigate whether basal cells were affected by prenatal smoke exposure, expression of gene markers *Trp63* and *Krt5*, selectively expressed in basal cells were investigated. However, gene expression of both basal markers were not different in both groups (data not shown).

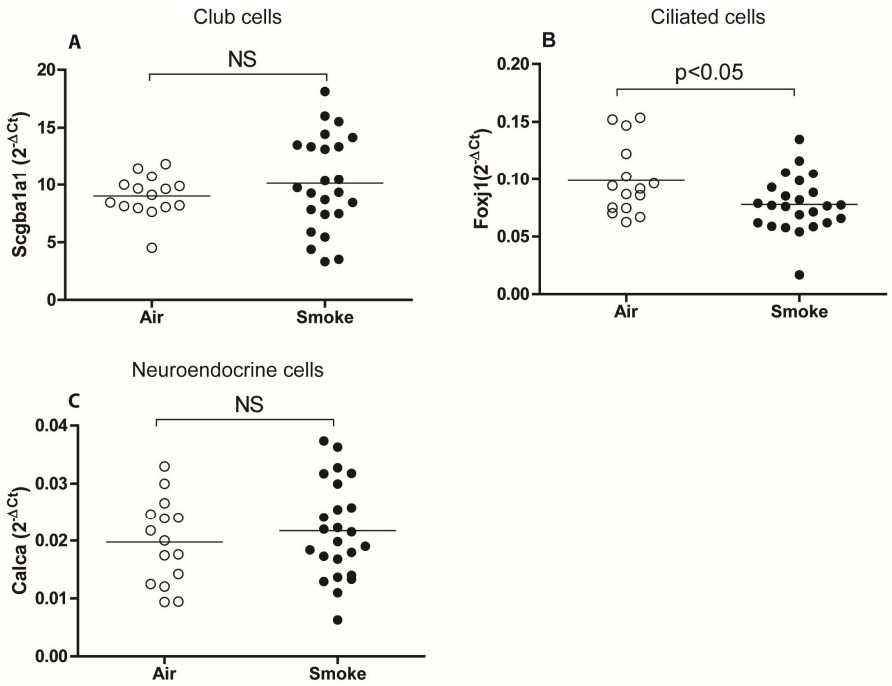


Figure 1. *Expression of the differentiation markers* Scgb1a1*, a marker for Club cells (A),* Foxj1*, a marker for ciliated cells (B) and* Calca*, a marker for neuroendocrine*

cells (C) was analyzed by real-time qPCR in RNA, isolated from lung tissue. Data represent medians of expression in neonatal pups, prenatally exposed to cigarette smoke or not. Foxj1*; Forkhead box J1.*

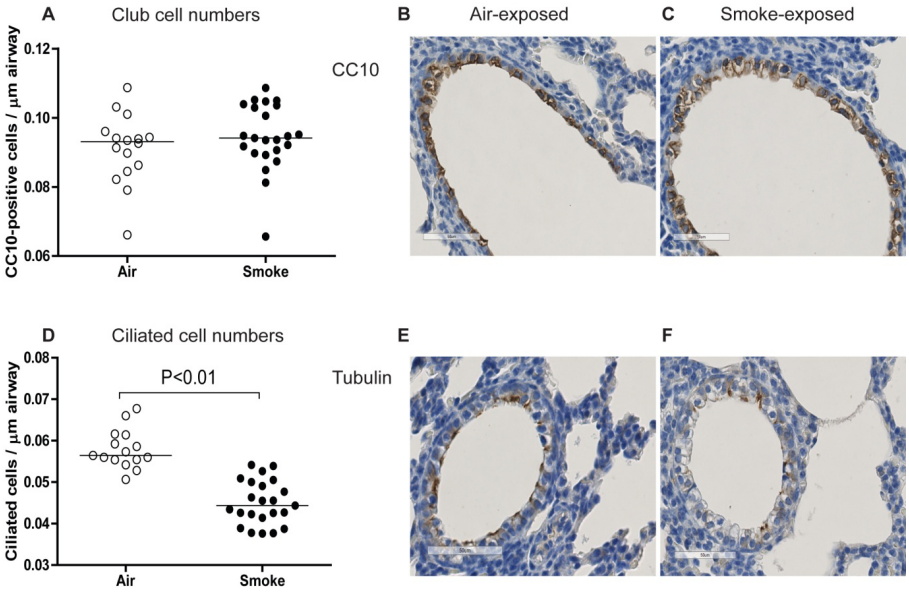


Figure 2. *Club cell numbers (A) and immunohistochemical staining of CC10 (Club cell 10 kD, brown, B and C) or ciliated cell numbers (D) and immunohistochemical staining of tubulin (brown, E and F) in airways in lung tissue from offspring of air-exposed mothers (B and E) or smoke-exposed mothers (C, F). Original magnification 40x.*

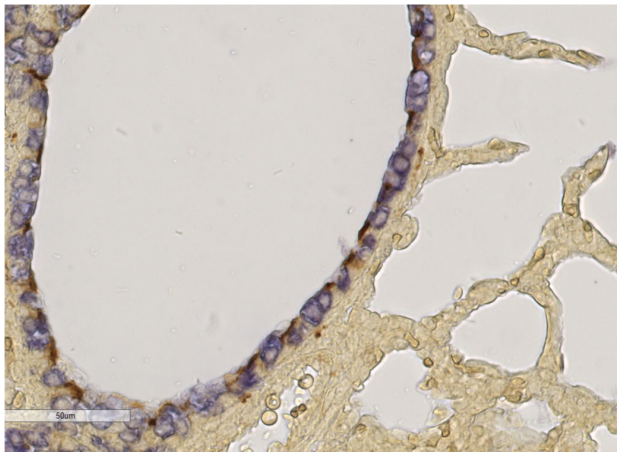


Figure 3. *Representative example of a double staining for CC10 (Club cell 10 kD, blue) and tubulin (brown).*

Maternal smoking during pregnancy induced goblet-cell related gene expression in offspring.

In order to further explain the previously observed HDM-induced goblet cell susceptibility in offspring from smoke-exposed mothers, genes important in goblet cell transformation were investigated. As shown in Figures 4a and b, offspring from smoke-exposed mothers had higher expressions of *Foxm1* ($p<0.05$) and *Spdef* ($p<0.001$). Expression of the genes *Nkx2-1, Foxa2, Foxa3, Hoxa5* and *Hoxb5, Muc5ac*, that are essential to maintain Club cell identity and/or inhibit *Spdef* function were either slightly decreased ($p=0.065$, *Nkx2-1*) or not affected (data not shown).

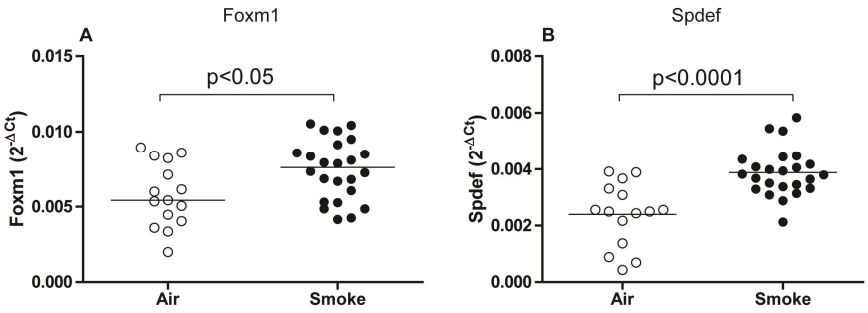


Figure 4. *Expression of genes that drive secretory cell development. Expression of the transcription factors* Foxm1 *(A) and* Spdef *(B), was analyzed by real-time qPCR in RNA, isolated from lung tissue. Data represent medians of expression in neonatal pups, prenatally exposed to cigarette smoke or not.* Foxm1*; Forkhead box M1,* Spdef*; Sam pointed domain-containing ETS transcription factor.*

Maternal smoking during pregnancy affected gene expression of Notch signaling pathway in offspring.

The Notch signaling pathway has been identified as a major regulator of specific cell fate in the developing and postnatal lung (Rock, et al. 2011; Tsao, et al. 2011). To further investigate whether Notch signaling was affected in prenatally smoke-exposed mice, gene expression of Notch

receptors *Notch 1, 2* and *3,* and gene expression of Notch target genes *Hey1, Hey2* and *Hes1*, important in epithelial cell differentiation, were investigated. As shown in Figure 5, offspring from smoke-exposed mothers had lower expression of *Notch1* (Figure 5a) and a higher expression of the Notch target gene *Hey1* (Figure 5b). The other members of this pathway that were investigated were not different (*Notch2, Hey2* and *Hes1*, Figure 5c-e) between both groups, or were too minimally expressed (*Notch3*, data not shown).

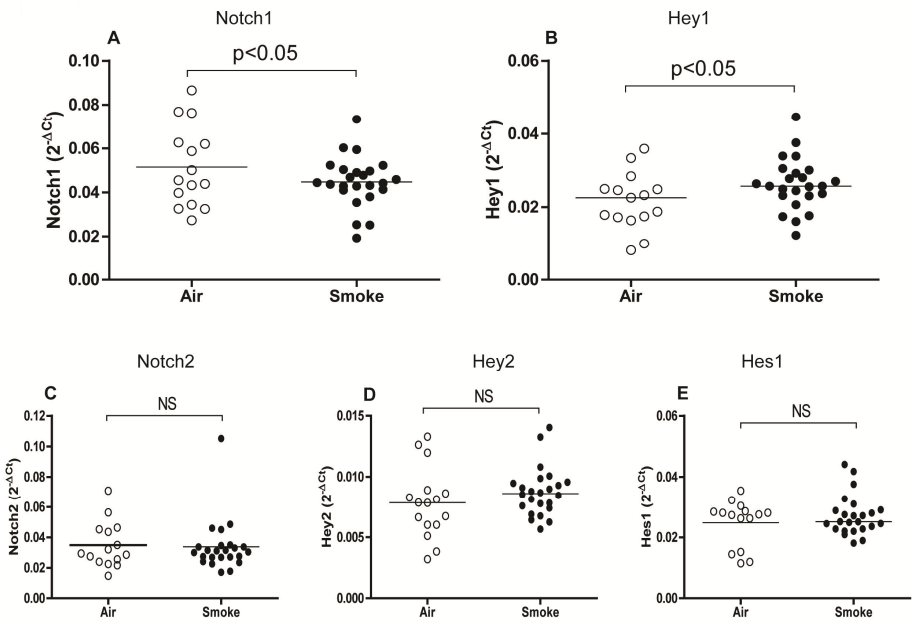


Figure 5. *Expression of genes of the Notch signaling pathway. Expression of* Notch1 *(A),* Hey1 *(B),* Notch2 *(C),* Hey2 *(D) and* Hes1 *(E) was analyzed by real-time qPCR in RNA, isolated from lung tissue. Data represent medians of expression in neonatal pups, prenatally exposed to cigarette smoke or not.* Hes1; *Hairy Enhancer of Split1,* Hey1*; Hes-related with YRPF motif1.*

Discussion

A number of studies by us and others have shown that smoking during pregnancy affects lung development and function in the offspring (Singh, et

al. 2003; Gaworski, et al. 2004; Gaworski, et al. 2004; Blacquiere, et al. 2009; Blacquiere, et al. 2010; Haley, et al. 2011; Manoli, et al. 2012). In the present study, the effect of maternal smoking during pregnancy on epithelial cell differentiation was investigated in neonatal offspring. Our three main observations in prenatally smoke-exposed offspring included: *lower* numbers of ciliated cells, *higher* expression of genes that are important in goblet cell differentiation, and lastly, altered expression of genes in the Notch signaling pathway.

This is the first study to show that prenatal smoke exposure is associated with downregulation of ciliated cells in the lung after birth. Ciliated cells are necessary for proper mucociliary clearance of particles and pathogens (Wanner, et al. 1996), and a decrease of ciliated cell numbers may account for a less effective clearance of pathogens from the lung. Indeed, epidemiological studies have shown that children born from a mother that smoked during pregnancy have a higher risk to suffer from airway infections (Broughton, et al. 2005). Our data is further supported by a recent *in vitro* study in which primary human bronchial epithelial cells were exposed to cigarette smoke extract (CSE) during differentiation at the air-liquid interface (Schamberger, et al. 2015). This CSE exposure was shown to reduce the number of ciliated cells, while it increased the number of Club cells and goblet cells. In addition FOXJ1, a master regulator in ciliogenesis and responsible for cilia length (You, et al. 2004; Brekman, et al. 2014), was studied. However, no changes in *FOXJ1* or *FOXJ1* target gene transcription upon CSE exposure was found, indicating that CSE influenced *FOXJ1*-independent processes crucial for ciliated cell fate, or affected ciliogenesis further downstream of *FOXJ1*. In our study, lower numbers of ciliated cells were accompanied by lower expression of *Foxj1*. *FOXJ1* expression was recently shown to be directly regulated by the Wnt/b-catenin signaling pathway (Caron, et al. 2012). This is consistent with our earlier findings where we demonstrated less Wnt/b-catenin signaling in the lungs of neonatal offspring from smoke-exposed mothers (Blacquiere, et al. 2010).

Our finding that reduced numbers of ciliated cells were not accompanied by the presence of more CC10 positive Club cells came somewhat as a surprise. As in the mouse, goblet cells have been shown to arise from Club cells (Hayashi, et al. 2004; Kouznetsova, et al. 2007; Chen, et al. 2009), more Club cells in offspring from smoking mothers could have been one explanation for our previous observation regarding increased HDM-induced goblet cell susceptibility in this group. Interestingly, however, several studies in a naphthalene toxicity model have reported the presence of a new subset of CC10 positive secretory cells. These so-called variant Club cells do not express cytochrome p450 (*Cyp2f2*), are naphthalene-resistant, and can self-renew and differentiate. They are located adjacent to neuroendocrine bodies of the airway (Hong, et al. 2001) or at the bronchoalveolar duct junctions (Giangreco, et al. 2002) to regenerate the damaged conducting airways (Reynolds, et al. 2000a; Reynolds, et al. 2000b). Whether prenatal smoke-exposure affects this cell population will be subject of further studies.

Another source for goblet cells could be the basal cell which in the mouse is mostly present in the trachea and the largest bronchi. Although expansion of the basal cell compartment is among the hallmark airway abnormalities of smokers and individuals with COPD (Demoly, et al. 1994; Khuri, et al. 2001), gene expression of two basal cell markers was not changed in offspring from smoking mothers.

Our second important observation was that prenatal smoke exposure increased expression of *Foxm1*, *Spdef* and decreased expression of *Nkx2-1* (trend). FOXM1, a transcription factor of the Forkhead box family, plays important roles during embryonic development, monocyte/macrophage recruitment, DNA repair, surfactant production and angiogenesis (Kalinichenko, et al. 2001; Kalin, et al. 2008; Balli, et al. 2013). Moreover, FOXM1 is known to promote SPDEF activity, a master transcription factor that regulates goblet cell differentiation in the airway epithelium (Park, et al. 2007). Therefore, higher expression of *Foxm1* could have contributed to the higher expression of *Spdef* in offspring from smoke-exposed mothers.

Interestingly, a trend for lower expression of *Nkx2-1* was observed in prenatally exposed offspring. NKX2-1 is a transcription factor that has been described to inhibit *Spdef* expression (Maeda, et al. 2011a), and therefore, also lower expression of NKX2-1 could have facilitated the higher expression of *Spdef* in prenatally smoke-exposed offspring. We found no differences in *Hoxa5* and *Hoxb5* expression. In *Hoxa5*-/- mice, the loss of *Hoxa5* function was shown to induce Club to goblet cell transdifferentiation. This was a Foxa2-independent process, accompanied by increased activity of Notch signaling (Boucherat, et al. 2012).

Our finding regarding the altered epithelial cell differentiation in prenatally smoke-exposed offspring does not support findings from a recent study in second hand smoke exposed offspring (Singh, et al. 2013). In that study, no change in ciliated cells was found, whereas the numbers of Club cells and *Cc10* gene expression were decreased. An explanation for these conflicting results could be that in the study from Singh *et al.* a different mouse strain was used (Balb/c), a different batch of cigarettes (2R1 research cigarettes), a different type of exposure (side stream instead of main stream) and a different smoking protocol (6 hrs exposure versus 2 times 50 minutes). Side stream smoke (SS) contains different concentrations of toxic components than mainstream smoke. For instance, SS contains ten times greater levels of polycyclic aromatic hydrocarbons than mainstream smoke (Weinberg, et al. 1989).

Notch signaling has been reported to promote secretory cell development over ciliary cell fate (Rock, et al. 2009; Rock, et al. 2011). Here we show that prenatal exposure to cigarette smoke upregulates *Hey1* expression. *Hey1* is a Notch target gene which implies active Notch signaling in offspring from smoke-exposed mothers. Therefore, active Notch signaling could have contributed to lower ciliated cell development and increased expression of goblet cell gene markers.

In conclusion, our studies indicate that smoking during pregnancy in mice changes epithelial cell differentiation and therefore increases risk for asthma in the offspring at two levels: (*i*) by inhibiting ciliated cell

differentiation, thereby increasing the risk to develop respiratory infections, which itself is a risk factor for asthma and (*ii*) by promoting secretory cell metaplasia through regulation of *Foxm1, Spdef* and *Nkx2-1*. These actions may be Notch-signaling related and provide insight into potential mechanisms underlying epidemiological observations on the association between maternal smoking and childhood or adolescent asthma.

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Conflict of interest

No competing interests declared

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Chapter 3

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

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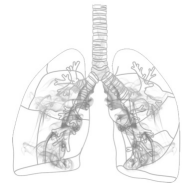

Abstract

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, antioxidant 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 was investigated by immunohistochemistry, whereas gene expression was assessed by quantitative real time PCR. We found that maternal smoking during pregnancy down regulated smooth muscle thickening and 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 transformation related protein P63 (*Trp63*) were higher in prenatally smoke-exposed mice. Offspring exposed to cigarette smoke for 12 weeks had more inflammation (M2 macrophage infiltration), remodeling (smooth muscle thickening), and expression of the mucus related gene *Muc5ac*, cytochrome P450 family 1 subfamily A member 1 (*Cyp1a1*) and AHR repressor (*Ahrr*) in lung. We conclude that prenatal smoke exposure affects gene expression from various pathways related to oxidative stress, lung injury and repair. Offspring smoking promotes tissue

remodeling, inflammation and tissue repair, which is not further enhanced by prenatal smoke exposure.

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 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 of 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, Trp63*). 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 with ARNT, and binding of AHRR/ARNT complex to DRE results in repression transcription of AHR's target genes.(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, as it can act as a cofactor of FOXO3, or a repressor of FOXO3 activity by deacetylation of the protein (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

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 C).

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/m^3 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=16 males and n=18 females) or smoke (n= 19 males and n=18 females) 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), *Trp63* (basal cell, Mm00495793_m1), and *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-ΔCp (Cp means crossing points). Undetectable Cp values of the genes of interest (>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, monoclonal 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 mm2 per mm airway or vessel in the total lung section.

M2 dominant macrophages were determined by double staining for MAC3 and YM1 (Polyclonal 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 Aldric) as chromogen. The number of MAC3 single positive and the number of MAC3-pposive/YM1-positive cells were counted manually in parenchymal lung tissue at x20 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 x8 magnification and averaged. Neutrophils (glutathione-disulfide reductase (GR1) , monoclonal rat anti GR1 antibody (BD Biosciences) were counted manually in a blinded manner at x20 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 SIRT (H-300, sc-15404, Santa Cruz). SIRT1 positive cells were counted at x20 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 was determined by staining formalin-fixed and paraffin-embedded lung sections with a polyclonal rabbit anti-mouse Keratin 5 antibody (ab52635, Abcam, Camebridge, UK).

Statistical methods

Results obtained from males are shown. Results of qRT-PCR and IHC are 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 effects 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 smoke-exposed mothers were more susceptible for smoke-induced inflammation, the presence of neutrophils, eosinophils, macrophages and M2 dominant macrophages was investigated. We found that the number of M2 dominant macrophages was increased in offspring that was postnatal 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 postnatal 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 postnatal smoke-exposed offspring (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).

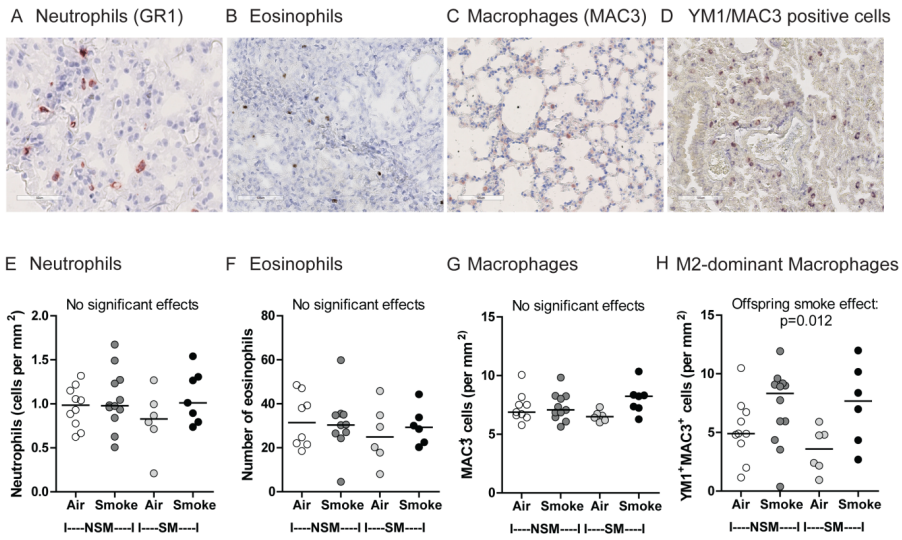

Figure 1 Infiltration of inflammatory cells in lung tissue from (prenatally) smoke exposed mice. *Representative pictures and scores of (A,E) Neutrophils (GR1), (B,F) Eosinophils (endogenous peroxidase activity), (C, G) Macrophages (MAC3) and (D, H) M2 dominant macrophages (YM1/MAC3). Original magnifications were x40 (A) and x20 (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.*

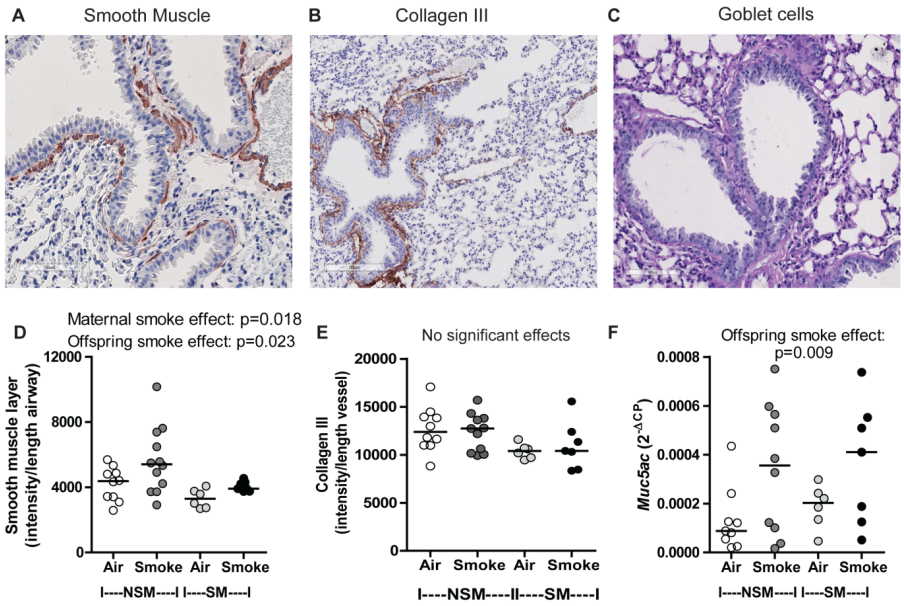

Figure 2 Airway remodeling in (prenatally) smoke-exposed mice. *Representative pictures and quantifications of (A,D) Smooth muscle actin (SMA) around the airways, (B,E) Collagen III deposition around blood vessels, (C) PAS negative airway epithelium and (F)* Muc5ac *mRNA expression after IHC or qRT-PCR 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.*

Next, gene expression 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 (Fig 3A, p=0.096). Offspring smoking additionally induced *Ahrr* and *Cyp1a1* gene expression, independent from prenatal smoke exposure (Fig 3B and 3C, p=0.047 and p=0.001, respectively).

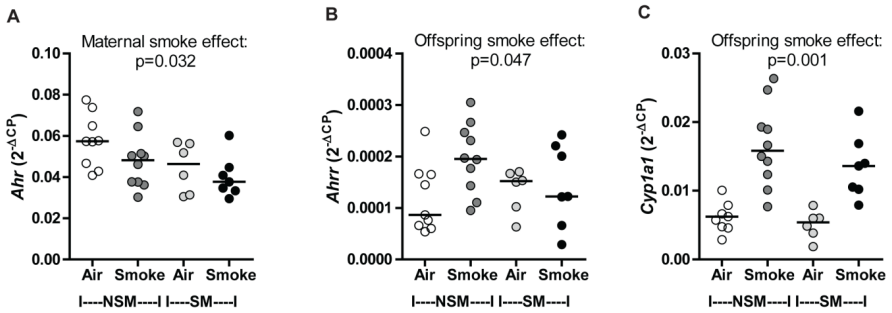

Figure 3 Expression of AhR signaling pathway related genes in (prenatally) smoke-exposed mice. *Expression of (A) Ahr, (B) Ahrr, and (C) Cyp1a1 was analyzed by qRT-PCR in RNA, isolated from lung tissue. Data represent medians of expression. NSM: air-exposed mother, SM: Smoke-exposed mother. The "Offspring 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 shows that prenatally 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* (r^2=0.78, p<0.0001, Fig 4C). Postnatal smoke-exposed mice had lower numbers of SIRT1 positive cells then air-exposed offspring (Fig 4D, 0.012). IHC staining of SIRT1 indicated that SIRT1 was expressed in the nucleus.

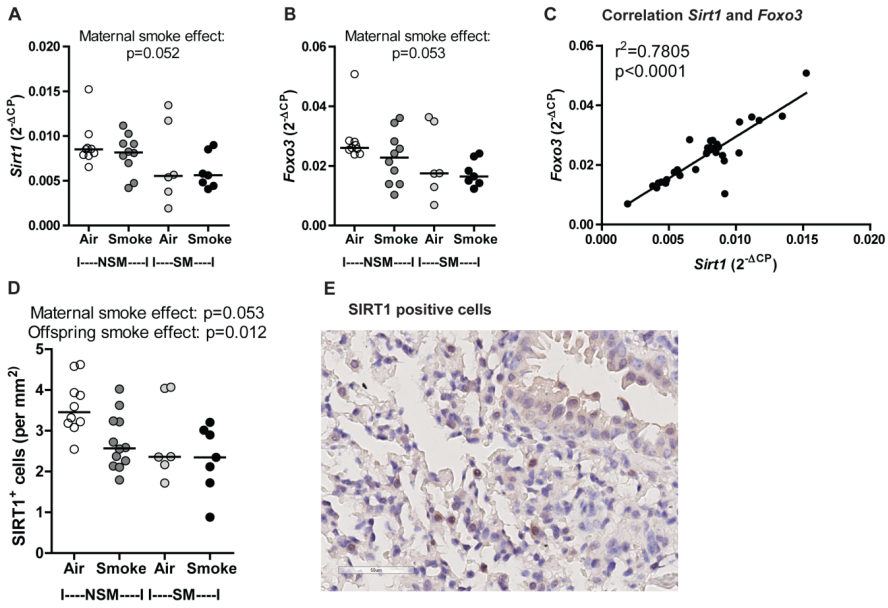

Figure 4 Lower expression of aging-related markers* Sirt1 *and* Foxo3 *in lungs of prenatally smoke-exposed mice. *Expression of (A)* Sirt1*, (B)* Foxo3*, (C) the correlation plot of* Sirt1 *expression versus Foxo3, and (D) the numbers of SIRT1 positive cells after qRT-PCR analysis and IHC staining of SIRT1 (Brown, E) in lung tissue from in lungs of prenatally smoke-exposed mice. Original magnification was x20. 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-Withney U-tests. 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 *Trp63*, 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 *Trp63* 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 are shown.

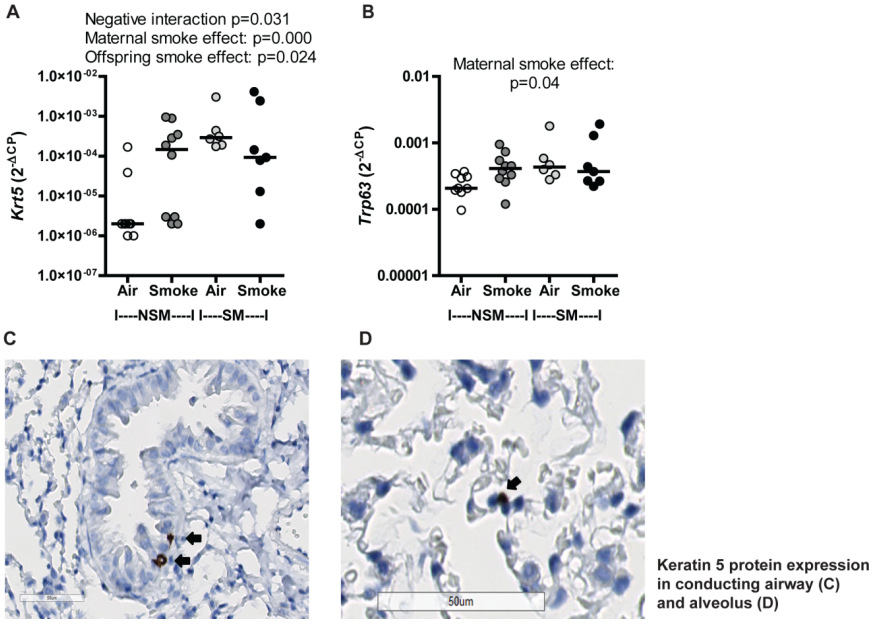

Figure 5 Higher expression of basal cell markers* Krt5 *and* Trp63 *in lungs of (prenatally) smoke-exposed mice. *mRNA expression of (A)* Krt5*, (B)* Trp63 *and Keratin 5 protein expression in a distal conducting airway (C) and alveolus (D) after IHC staining of Keratin 5 (brown) in lungs of prenatally smoke-exposed mice. Original magnifications were x20 (C) and x40 (D). Arrows indicate the Keratin 5 positive cells. 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. (A) Negative interaction maternal smoke and offspring smoke p=0.033 from linear regression analyses indicates that the offspring smoke exposure effect on increased Krt5 gene expression was weaker in offspring from smoking mothers*

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 immune cell regulation (*Ahr*), the cellular stress response, aging and apoptosis (*Foxo3* and *Sirt1*) as well as tissue repair (*Krt5* and *Trp63*). 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 gene *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 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 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 to investigate 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 was associated with important risk increases of subsequent lung cancer (50). In addition, also increased CYP1A1 activity and expression has linked CYP1A1 recently to development of tobacco related lung cancer (51). Besides that, numerous studies have

implicated a role for 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 that are largely being 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 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 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 dysregulated 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 *Trp63* gene expression in lungs from 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 in the effects of (prenatal) smoke exposure in our model.

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 *Trp63* 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 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 differential expressed genes are necessary for a more in depth insight in the effect of prenatal smoke exposure on cigarette smoke-induced lung pathology later in life.

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Chapter 4

Aberrant DNA methylation and expression of *SPDEF* and *FOXA2* in airway epithelium of patients with COPD

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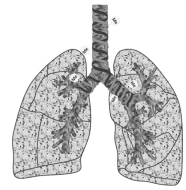

Abstract

Goblet cell metaplasia, a common feature of chronic obstructive pulmonary disease (COPD) is associated with mucus hypersecretion which contributes to the morbidity and mortality of the patients. Transcription factors SAM-pointed domain–containing Ets-like factor (SPDEF) and forkhead box protein A2 (FOXA2) dictate goblet cell differentiation. This study aimed to: 1) investigate DNA methylation and expression of *SPDEF* and *FOXA2* during goblet cell differentiation and 2) compare between epithelial cells from individuals with COPD and controls.

To assess DNA methylation and expression of *SPDEF* and *FOXA2* during golet cell differentiation (Aim 1), primary airway epithelial cells isolated from trachea (controls) and bronchial tissue (individuals with COPD) were differentiated by culture at the air-liquid interface (ALI) in the presence of cytokine interleukin (IL)-13 to promote the goblet cell differentiation. We found that *SPDEF* expression was increased during goblet cell differentiation, while *FOXA2* expression was decreased. Importantly, CpG #8 in the *SPDEF* promoter was hypermethylated upon differentiation, while DNA methylation of *FOXA2* promoter was not changed.

In the absence of IL-13, we found that COPD-derived ALI cultures displayed a higher *SPDEF* expression than control-derived ALI cultures, whereas no difference for *FOXA2* expression (Aim 2). This was accompanied with hypomethylation of CpG #6 in the *SPDEF* promoter and also hypomethylation of CpG #10 and #11 in the *FOXA2* promoter. These findings suggest that aberrant DNA methylation of *SPDEF* and *FOXA2* might be one of the factors underlying increased mucus hypersecretion in COPD, opening new avenues for epigenetic-based inhibiting of mucus hypersecretion.

Keywords: SPDEF, *FOXA2*, DNA methylation, mucus, COPD

Introduction

Chronic bronchitis, one of the clinical phenotypes of chronic obstructive pulmonary disease (COPD), is characterized by goblet cell metaplasia and mucus hyperproduction which contributes to the morbidity and mortality of patients [1-3]. The tracheobronchial epithelium of the human airways consists of basal cells, ciliated cells, club (Clara) cells, goblet cells and neuroendocrine cells [4,5]. Basal cells serve as the progenitor cells from which goblet cells and ciliated cells are derived, both in the normal airway epithelia renewal process and during abnormal remodeling in disease [6,7]. Goblet cell differentiation is dictated by a large network of genes, in which transcription factors SAM-pointed domain–containing ETS-like factor (SPDEF) and forkhead box protein A2 (FOXA2) are two key regulators. SPDEF is required for the goblet cell differentiation and mucus production, including the major secreted airway mucin MUC5AC (mucin 5AC) [8-10], whereas FOXA2 is a potent inhibitor of goblet cell differentiation in the lung [11-13]. Recent studies have shown that *SPDEF* is higher expressed (mRNA) in the large airway epithelium of smokers compared to nonsmokers [14,15] and also higher expressed (protein) in lung tissue of patients with asthma and COPD compared to healthy controls [16]. FOXA2 was shown to be reduced (both mRNA and protein) and was negatively correlated with MUC5AC in bronchial epithelium of patients with asthma [17] and in nasal tissue of patients with chronic rhinosinusitis [18]. In addition, *FOXA2* was lower expressed (mRNA) in small airway epithelium of both healthy smokers and COPD smokers than in non-smokers [19].

DNA methylation is an important mechanism in the regulation of gene expression during adult stem cell renewal and differentiation, as is shown for the differentiation of hematopoietic, epidermal and intestinal stem cells [20-23]. However, the role of DNA methylation in airway basal cell differentiation has not been evaluated. Other studies showed that aberrant DNA methylation was associated with dysregulation of *SPDEF* and *FOXA2* expression in lung cancer [24,25], although DNA methylation regulation of

SPDEF and *FOXA2* expression has not been assessed for lung tissues of patients with COPD.

In this study we aimed to investigate the DNA methylation and expression of *SPDEF* and *FOXA2* during goblet cell differentiation, and further identify whether DNA methylation and expression of *SPDEF* and *FOXA2* are different in patients with COPD compared to control subjects after *in vitro* airway epithelial cell differentiation. Expression of *SPDEF* target genes *MUC5AC* and Anterior gradient 2 (*AGR2*), and the ciliated cell related gene Forkhead Box J1 (*FOXJ1*) were additionally assessed.

Materials and Methods

Ethics statement

The study protocol followed national ethical and professional guidelines ('Code of conduct; Dutch federation of biomedical scientific societies'; http://www.federa.org) for all lung tissue and explant cell culture studies in Groningen.

Culture of PBEC cells

Primary human bronchial epithelial cells (PBECs) were obtained from bronchial tissue harvested from transplant recipient lungs of 18 patients with GOLD (Global Initiative for Chronic Obstructive Lung Disease) stage IV COPD, and residual tracheal and main stem bronchial tissue from 17 transplant donors (controls). No information was available from the transplant donors. Characteristics of patients with COPD and details on the experimental design are shown in table 1.

Table 1 Characteristics of Subjects and experiments design

	Age (yr)	Sex	smoking status	Pack-Years	FEV1%pred	FEV1/FVC%	RNA	DNA
COPD 1*	56	m	ex	30	31	29	√	√
COPD 2*	59	f	ex	41	87	59	√	√
COPD 3*	60	f	ex	20	33	38	√	√
COPD 4*	56	f	ex	30	14	25	√	√
COPD 5*	61	f	ex	26	21	28	√	√
COPD 6	57	f	ex	40	18	25	×	√
COPD 7	61	f	ex	35	19	23	×	√
COPD 8	55	f	ex	18	19	52	×	√
COPD 9	49	m	ex	11	20	22	√	√
COPD 10	60	m	ex	23	16	29	√	√
COPD 11	48	m	ex	27	12	23	×	√
COPD 12	58	f	ex	38	60	46	√	√
COPD 15	64	m	non	0	39	53	√	√
COPD 16	53	m	ex	40	25	25	√	×
COPD 17	57	m	ex	30	11	31	√	×
COPD 18	57	f	ex	45	23	24	√	×
Control 1*	NA	NA	NA	NA	NA	NA	√	√
Control 2*	NA	NA	NA	NA	NA	NA	√	√
Control 3*	NA	NA	NA	NA	NA	NA	√	√
Control 4*	NA	NA	NA	NA	NA	NA	√	√
Control 5*	NA	NA	NA	NA	NA	NA	√	√
Control 6*	NA	NA	NA	NA	NA	NA	√	√
Control 7	NA	NA	NA	NA	NA	NA	×	√
Control 8	NA	NA	NA	NA	NA	NA	×	√
Control 9	NA	NA	NA	NA	NA	NA	×	√
Control 10	NA	NA	NA	NA	NA	NA	√	√
Control 11	NA	NA	NA	NA	NA	NA	√	√
Control 12	NA	NA	NA	NA	NA	NA	×	√
Control 13	NA	NA	NA	NA	NA	NA	√	×
Control 14	NA	NA	NA	NA	NA	NA	√	√
Control 15	NA	NA	NA	NA	NA	NA	√	×
Control 16	NA	NA	NA	NA	NA	NA	√	×
Control 17	NA	NA	NA	NA	NA	NA	√	×

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; m = male; f = female; ex = ex-smoker; non = non-smoker; NA = not asked for; FEV1%pred = forced expiratory volume during the first second as percentage of predicted; FEV1/FVC% = the ratio of FEV1 to FVC (Forced vital capacity); ALI = air-liquid interface culture; IL-13 = interleukin-13.

*Cells were cultured at ALI in the presence of IL-13 and harevested after 0, 14, 21 and 28 days; the rest cells were cultured at ALI without the presence of IL-13 and harvested after 14 days.

For the initial experiments, the third passage PBECs from control subjects 1-6 and patients with COPD 1-5 (Table 1) were cultured in bronchial epithelium growth medium (BEGM, Lonza, Walkersville, MD, USA) until confluence on fibronectin/collagen pre-coated transwell inserts (0.4 µm pore size, 12 mm diameter; Corning, NY, USA), and were allowed to differentiate at Air-Liquid Interface (ALI) culture in the presence of interleukin (IL)-13 (1 ng/ml; Peprotech, Rocky Hill, NJ, USA) to enhance

goblet cell differentiation (Fig. 1a) as previously described [26]. Cells were harvested after 0, 14, 21 or 28 days of air exposure for the analysis of the mRNA, morphology and DNA methylation. For the latter experiments, the third passage PBECs from control subjects 7-17 and COPD patients 6-18 (Table1) were cultured in BEGM medium until confluence on pre-coated transwell inserts (0.4 μm pore size, 6.5 mm diameter; Corning), and allowed to differentiate at ALI culture without IL-13 (Fig. 4a) as previously described [27]. Cells were harvested after 14 days of air exposure for the analysis of the mRNA and DNA methylation.

For morphology analyses in transverse, the transwell inserts were formalin-fixed and embedded in paraffin, according to the Corning's instructions, then cross-sections (5 μm thick) were analyzed with immunohistochemistry staining; for morphology analyses in horizontal, transwell inserts were fixed with 4% (w/v) paraformaldehyde (Merck, Darmstadt, Germany), after which the membrane was cut into four quarters and analyzed with immunohistochemistry staining.

Detection of goblet cells and ciliated cells with immunohistochemistry staining

Paraffin sections and inserts membranes were stained for ciliated cells or goblet cells using standard immunohistochemical procedures. To determine the presence of ciliated cells, slides were stained incubated with a monoclonal mouse anti-acetylated α-tubulin antibody (Sigma-Aldrich T7451, St. Louis, MO, USA) and visualized with Diaminobenzidine (DAB, Sigma) solution. Goblet cells were stained with Alcian Blue (together with the previously described visualization of ciliated cells), or stained with a monoclonal mouse anti-MUC5AC antibody (Abcam, ab3649, Cambridge, UK) and visualized with 3-amino-9 ethylcarbazole (AEC, Sigma)

mRNA expression by quantitative real-time PCR

Total RNA from PBEC was extracted using Trizol reagent (Thermo Fisher Scientific, Carlshad, USA), according to the manufacturer's instructions.

Then, 500 ng of total RNA was used for cDNA synthesis with random primers using Superscript II RNase H - Reverse transcriptase (Thermo Fisher Scientific). *SPDEF, MUC5AC, AGR2, FOXA2, FOXJ1* and *GAPDH* expression was quantified using 5 ng cDNA, qPCR MasterMix Plus (Eurogentec, Belgium) and Taqman gene-specific primer/probes (*SPDEF*: Hs01026050_m1; *MUC5AC*: Hs00873651_Mh; *AGR2*: Hs00356521_m1; *FOXA2*: Hs00232764_m1; *FOXJ1*: Hs00230964_m1; *GAPDH*: Hs02758991_g1, Thermo Fisher Scientific) for 40 cycles with LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). Data were analyzed with LightCycler® 480 SW 1.5 software (Roche) and the Fit points method, according to the manufacturer's instructions. Expression levels relative to *GAPDH* were determined with the formula $2^{-\Delta Cp}$ (Cp means crossing points). Samples for which no amplification could be detected were assigned a Cp value of 40 (the total number of PCR cycles).

Methylation analysis by pyrosequencing

For DNA methylation analysis of the target regions, genomic DNA was extracted with chloroform-isopropanol and bisulfite converted using the EZ DNA Methylation-Kit (Zymo Research), following the manufacturer's protocol. Bisulfite-converted DNA (10-20ng) was amplified by PCR in a 25 µl reaction using the Pyromark PCR kit (Qiagen). Pyrosequencing was performed on the Pyromark Q24 pyrosequencer (Qiagen) according to the manufacturer's guidelines, using a specific sequencing primer. Analysis of methylation levels at each CpG site was determined using Pyromark Q24 Software (Qiagen). The pyrosequencing primers information is presented in Supplementary Table S1.

Statistics

Results obtained from qRT-PCR and pyrosequencing are expressed as median and range respectively. For comparisons between undifferentiated and differentiated epithelial cells, the Kruskal-Wallis nonparametric test with Dunn's posttest was applied, data from day 0 was compared to other days of differentiation (day 14, 21, and 28). For comparisons of expression

levels between COPD and controls, the Mann-Whitney U-test was applied. Correlation analyses of the level of methylation level and mRNA within the same sample was tested by Spearman nonparametric correlation test. All stastistical analyses were performed with Prism v5.0 (GraphPad software, San Diego, CA, USA). Differences at $P<0.05$ were considered to be statistically significant.

Results

SPDEF and *FOXA2* expression profiles during epithelial cell differentiation in the presence of IL-13

First, in order to validate the role of transcription factors *SPDEF* and *FOXA2* in goblet cell differentiation, PBECs from six control individuals (Table 1, Control subjects 1-6) were ALI-cultured for 28 days in the presence of IL-13 to promote goblet cell differentiation (Fig. 1a). At days 0, 14, 21 and 28, the differentiation state of PBEC was characterized by immunohistochemistry staining and quantitative real-time PCR. As expected, this protocol induced the differentiation of goblet cells as shown by the Alcian Blue positive cells and MUC5AC positive cells after 14 days to 28 days of ALI culture (Fig. 1b). Immunohistochemistry staining demonstrated that approximately 5% of the cells represented goblet cells, whereas the majority of cells consisted of ciliated cells (tubulin positive) or other cells (negative for both alcian blue and tubulin).

Goblet cell differentiation was accompanied by increased expression of *MUC5AC* (59.6 fold) and Anterior gradient 2 (*AGR2*) (4.5 fold), which encodes a potential chaperone required for mucin packaging (Fig. 1c). These changes were observed after 14 days of ALI culture compared to expression at day 0, and the expression levels remained consistent after 21 and 28 days of ALI culture (Fig. 1c). As expected, increased *MUC5AC* expression was accompanied by increased expression of transcription factor *SPDEF* (2.8 fold at day 21, 2.2 fold at day 28) and decreased

expression of *FOXA2* (to 28.6% of the start level at day 21, to 19.7% at day 28) (Fig.1d). In line with the differentiation of ciliated cells, increased expression of the ciliated cell marker Forkhead Box J1 (*FOXJ1*, a key transcription factor for ciliated cell differentiation) was also found (Fig.S1).

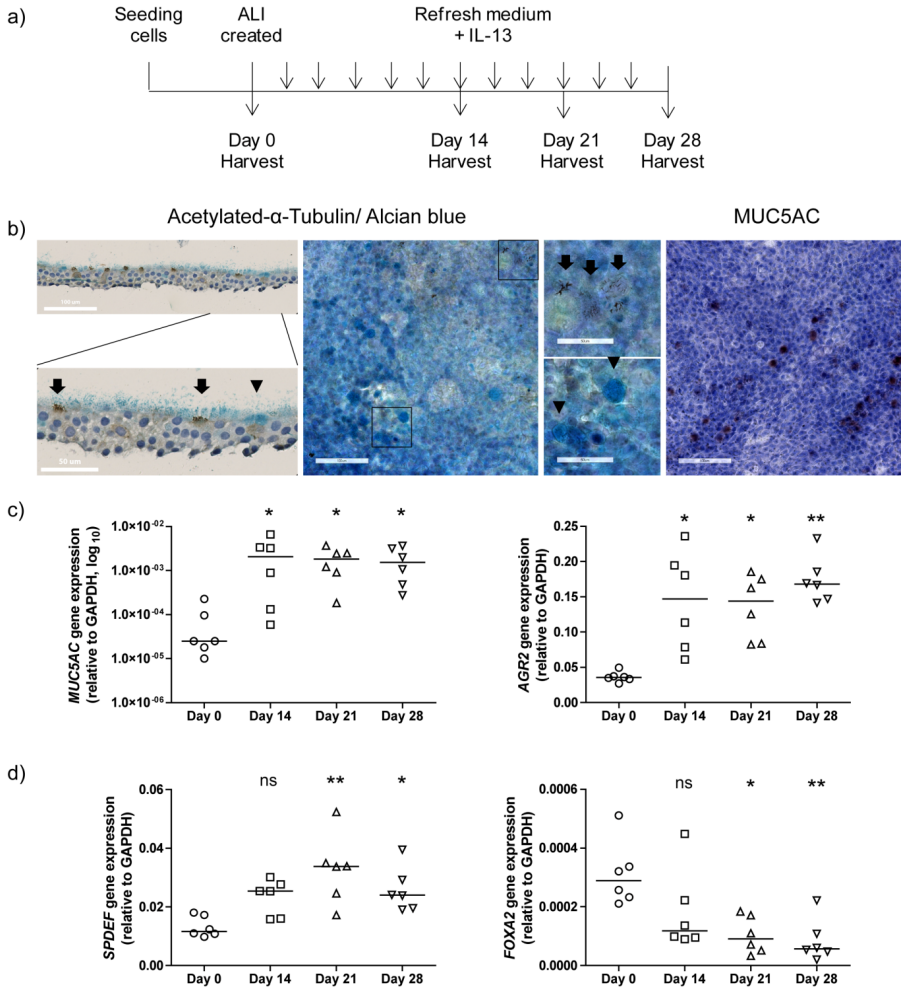

Figure 1 Characterization of the differentiation state of primary bronchial epithelial cells (PBEC) cultured in the air-liquid interface (ALI) model. *a) Schematic representation of the ALI culture model. PBECs were seeded on to a transwell insert and grown until confluence. Thereafter, apical medium was removed to create an ALI. Medium and stimuli were refreshed three times per week. Cells were harvested after 0, 14, 21, or 28 days for RNA, DNA and morphology analysis. b-d) PBEC from control subjects 1-6 (Table 1, n=6) were cultured at ALI with IL-13 stimulation. b)*

Representative images of immunohistochemistry staining on the differentiated ciliated cells and goblet cells at ALI day 21. Ciliated cells were determined by acetylated-α-tubulin antibody staining and specified by arrows in the images; goblet cells were determined by alcian blue staining and MUC5AC antibody staining, and were specified by arrow heads in the images. c) mRNA expression of MUC5AC *and* AGR2 *were analysed by real-time quantitative PCR at four different time points. d) mRNA expression of* SPDEF *and* FOXA2 *were analysed. Medians are indicated. Significance was tested by the Kruskal-Wallis nonparametric test with Dunn's posttest data. ns not significant, *p<0.05, and **p<0.01; data from day 14, 21, and 28 were compared to day 0.*

DNA methylation dynamics within *SPDEF* and *FOXA2* promoter during IL-13-induced goblet cell differentiation

Next, DNA methylation of *SPDEF* and *FOXA2* was assessed in the total cell population at different time points during goblet cell differentiation (Table1, Control subjects 1-6). Fourteen CpG sites within the *SPDEF* promoter were analyzed at five loci (Fig. 2a, loci A to E) using pyrosequencing analysis. Compared to day 0 of ALI culture, methylation levels of CpG sites #3 and 8 increased significantly at day 14 (CpG #3: from 88% to 93%, CpG #8: from 16% to 27%) and the methylation level of CpG #8 remained higher after 21 days and 28 days of ALI culture (Fig. 2b). Importantly, the methylation level in CpG #8 was positively correlated with mRNA expression of *SPDEF* (Spearman r= 0.567, p<0.004, Fig S2). CpG #15 was the only CpG site of which methylation was decreased at day 28 of ALI culture compared to day 0 (from very low level of 3% at day 0 to 2% at day 28, Fig 2b).

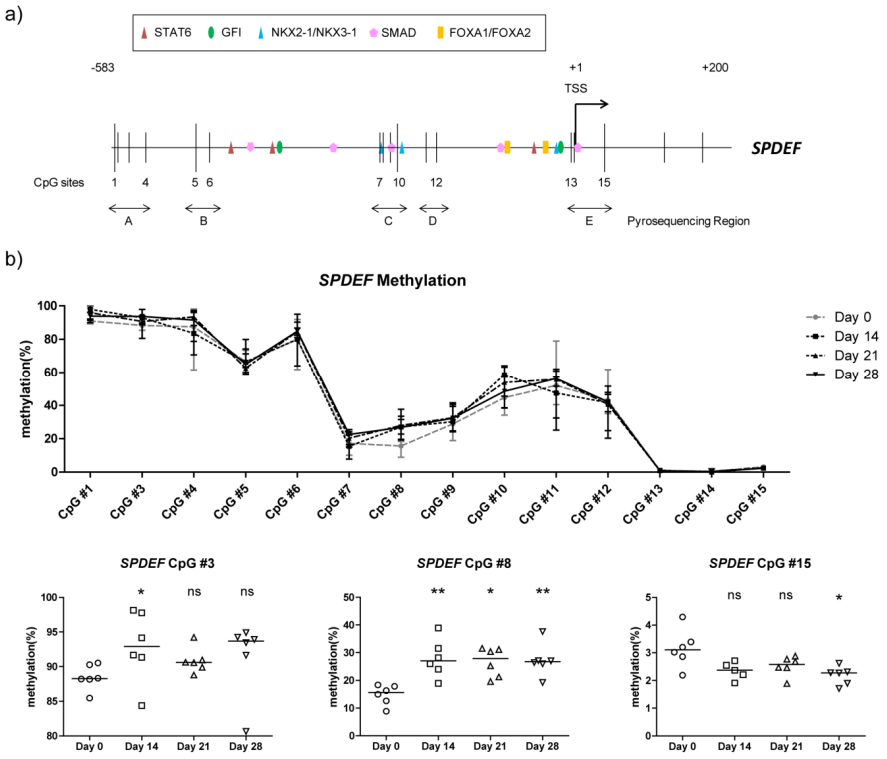

Figure 2 Dynamic changes of DNA methylation in the SPDEF promoter during goblet cells differentiation of PBEC from control subjects. *a) Schematic representation of the promoter region of the SPDEF gene, outlining the putative binding sites for the most relevant transcription factors (STAT6, GFI, NKX2-1/NKX3-1, SMAD, and FOXA1/FOXA2) as analyzed using MatInspector software. The transcription start site was shown as +1. CpGs are indicated as vertical bars. DNA methylation status of 15 CpGs was analyzed using pyrosequencing for the indicated areas. b) PBEC from control subjects1-6 (Table 1, n=6) were cultured at ALI and DNA methylation levels were analysed at four different time points. Data represent the connected median methylation levels (Min to Max) of different CpG sites at each time points, and differential methylated CpG sites are specified with medians indicated. Significance was tested by the Kruskal-Wallis nonparametric test with Dunn's posttest data. ns not significant, *p<0.05, and **p<0.01; data from day 14, 21, and 28 were compared to day 0.*

For *FOXA2*, six CpG sites that were part of a CpG Island in the promoter were examined (Fig. 3a, CpG #10-15). Methylation in these sites did not change despite of the observed *FOXA2* down regulation during differentiation (Fig. 3b).

In order to also investigate goblet cell differentiation in COPD, primary cells from five patients with COPD (Table1, COPD patients 1-5) were cultured at ALI in the presence of IL-13 using the same protocol. Similar to the observations in cells from controls, increased expression of the goblet cell markers (*MUC5AC*, *AGR2* and *SPDEF*) and the ciliated cell marker (*FOXJ1*) were observed after 14 to 28 days of ALI culture (Fig. S3a). In addition, a same trend of DNA methylation dynamics in the *SPDEF* promoter was observed (Fig. S4a) and the methylation level of CpG #8 was positively correlated with mRNA expression of *SPDEF* (Spearman r= 0.6165, p<0.004, Fig. S4b). However, different from the observations in control, *FOXA2* expression was not decreased during the IL-13-induced goblet cell differentiation of cells from patients with COPD (Fig. S3b), whereas loss of DNA methylation in the *FOXA2* promoter was observed (CpG #14: from 12% at day 0 to 8% at day 28, CpG #15: from 15% at day 0 to 11% at day 28, Fig. S5).

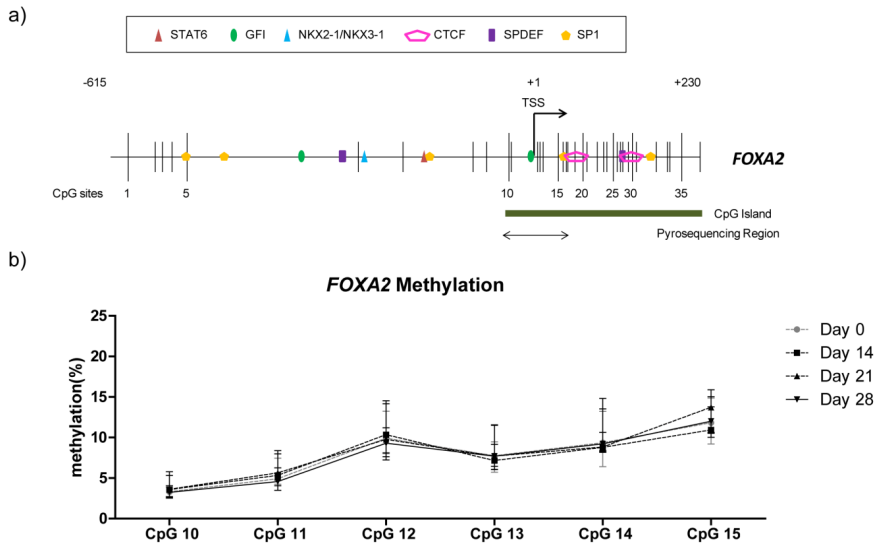

Figure 3 Dynamic changes of DNA methylation in the* FOXA2 *promoter during goblet cells differentiation of PBEC from control subjects. *a) Schematic representations of the promoter region of the* FOXA2 *gene, outlining the putative binding sites for transcription factors (STAT6, GFI, NKX2-1/NKX3-1, CTCF, SPDEF, and SP1) (MatInspector, top relevants). DNA methylation levels of six CpG sites in the promoter, which were part of a CpG island (green box), were examined for the*

indicated areas. b) PBEC from control subjects1-6 (Table 1, n=6) were cultured at ALI and DNA methylation levels were analysed at four different time points. Data represent the connected median methylation levels (Min to Max) of different CpG sites at each time points.

Aberrant expression and DNA methylation of *SPDEF* and *FOXA2* in airway epithelial cells of COPD

In order to investigate putative differences in airway epithelial cell differentiation between COPD and control, PBECs from COPD and control subjects (Table 1, COPD patients 6-18 and Control subjects 7-17) were differentiated at ALI-culture in absence of IL-13, and gene expression and DNA methylation levels were compared in the total cell population after 14 days of ALI culture (Fig. 4a). We found that epithelial cells derived from patients with COPD had significantly higher mRNA expressions of *MUC5AC*, *AGR2* and *SPDEF* than epithelial cells from controls (*MUC5AC*: 21.1 fold, *SPDEF*: 1.9 fold, *AGR2*: 2.9 fold, Fig. 4b), whereas no difference was found in *FOXA2* and *FOXJ1* expression between COPD-derived cells and control-derived cells (Fig. 4b and S6).

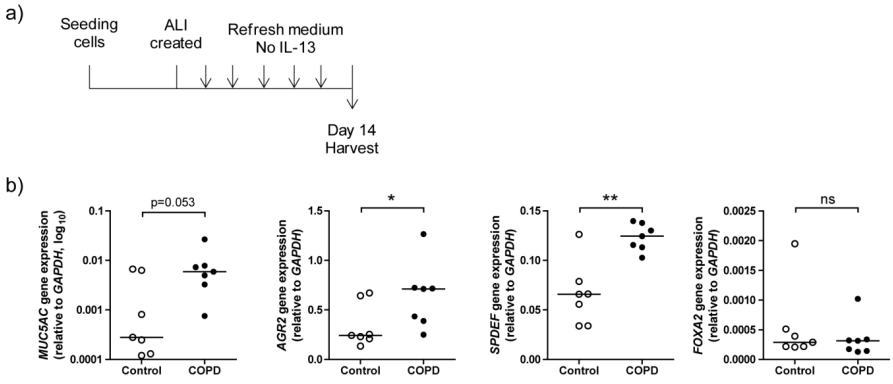

Figure 4 Differential mRNA expression profiles in the PBECs from COPD patients and control subjects. *a) Schematic representation of the ALI culture model. PBECs from control 7-17 and COPD 6-18 (Table 1) were seeded onto transwell inserts and grown to confluence. Thereafter, apical medium was removed to create an ALI. Medium were refreshed three times per week. Cells were harvested after 14 days for RNA and DNA analysis. b) Expression of* MUC5AC*,* AGR2*,* SPDEF *and* FOXA2 *were*

*analyzed by real-time quantitative PCR. Significance was tested by the Mann-Whitney U test. Medians are indicated. ns not significant, *p<0.05, and **p<0.01.*

COPD-derived cells presented significant lower methylation levels of CpG site #6 in the *SPDEF* promoter (85% in control, 73% in COPD, Fig. 5a) and also lower methylation levels of three CpG sites in the *FOXA2* promoter (CpG #10: 4% in control and 2% in COPD, CpG #11: 5% in control and 3% in COPD, CpG #15: 13% in control and 9% in COPD, Fig. 5b) than control-derived cells at ALI-day 14.

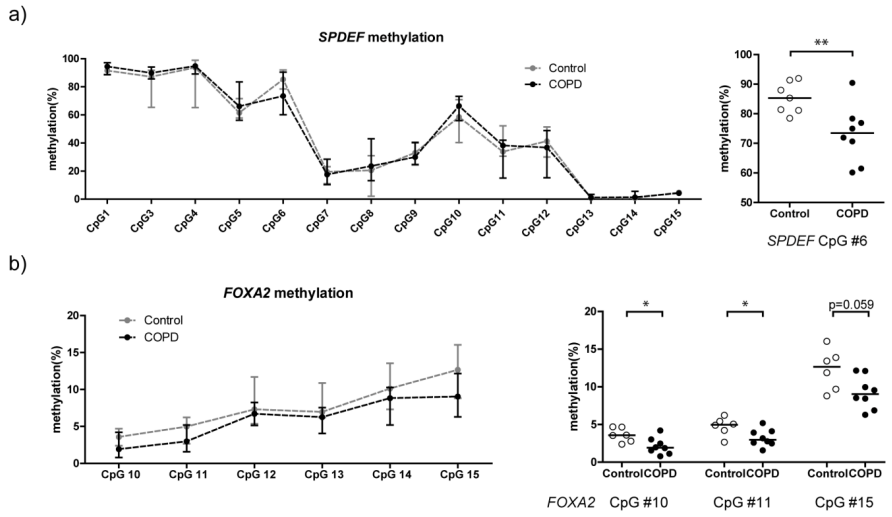

Figure 5 Differential DNA methylation of* SPDEF *and* FOXA2 *in the PBECs from COPD patients and control subjects. *PBECs from control 7-17 and COPD 6-18 (Table 1) were differentiated at ALI for 14 days, and DNA methylation of a)* SPDEF *and b)* FOXA2 *was analyzed by pyrosequencing. Data represent the connected median methylation levels (Min to Max) of different CpG sites in controls or in COPD, and differentiated methylated CpG sites between COPD-derived culture and control-derived culture were shown. Significance was tested by the Mann-Whitney U test. Medians are indicated. ns not significant, *p<0.05, and **p<0.01.*

Discussion

The two main observations in our study are that A) *SPDEF* expression in airway epithelium was induced during the IL-13-induced goblet cell differentiation and its expression was correlated with altered DNA methylation in epithelial cells, derived from both controls and patients with COPD, and B) *SPDEF* and *MUC5AC* were higher expressed in ALI-differentiated epithelial cells from patients with COPD compared to controls, which was accompanied with DNA hypomethylation in the *SPDEF* promoter. Furthermore, *FOXA2* expression was not affected during airway epithelial cell differentiation in patients with COPD, but was decreased during differentiation in controls, which was not accompanied by changes in DNA methylation.

SPDEF has been described, by us and others, to be up regulated in human bronchial epithelial cells in response to IL-13 [26,28]. Our study confirms the increased *SPDEF* expression after IL-13 stimulation, both in epithelial cells from controls and patients with COPD. During this process, *SPDEF* up regulation was accompanied by dynamic changes of methylation at several CpG sites of the *SPDEF* promoter. Importantly, methylation of CpG#8 was consistently and positively correlated to SPDEF expression in the IL-13-induced goblet cell differentiation of cells from both controls and patients with COPD. This is of interest as CpG#8 locates in a putative binding site for transcription factor NK2 homeobox 1 (NKX2-1). NKX2-1 is an airway epithelial-specific transcription factor which has been found to inhibit SPDEF expression and prevent ovalbumin-induced goblet cell differentiation and lung inflammation in transgenic *NKX2-1* overexpressing mice [29]. Interestingly, NKX2-1 was found to be methylation sensitive in the regulation of its target gene Surfactant Protein B and myosin binding protein H [30,31]. The hypermethylation of CpG #8 in the *SPDEF* promoter might prevent binding of NKX2-1, leading to increased transcription of *SPDEF* during differentiation. We speculate that NKX2-1 is a transcription inhibitor of *SPDEF* and that DNA methylation impairs the NKX2-1 inhibitory effect on the *SPDEF* promoter activity. For now, there is no direct evidence

showing that *SPDEF* is a target gene of NKX2-1 except for their inverse role in goblet cell differentiation and mucus production. It will be interesting to further investigate the relation of NKX2-1 binding and *SPDEF* promoter methylation (particularly CpG #8) to *SPDEF* promoter activity in the future.

SPDEF has previously been shown to be highly expressed in bronchial epithelium of smokers and patients with COPD [14-16], which is in agreement with our findings. Increased expression of *SPDEF*, *MUC5AC* and *AGR2* was found in COPD-derived ALI cultures when compared to controls. Moreover, our data demonstrate that there was hypomethylation of CpG #6 in the *SPDEF* promoter in the COPD-derived ALI cultures, which is in line with the over expression and hypomethylation of *SPDEF* in lung cancer [24]. There are some confounding factors that may have contributed to the difference we found between COPD and control. Besides genetic differences and lack of information on medication, no information exists on whether the controls have been smoking. In addition, the location of sampling of the primary cells from control and COPD was not exactly the same. Primary cells from COPD were obtained from the large bronchus, whereas samples from transplant donor controls were obtained from the trachea/ main stem area. However, goblet cell density is decreased from proximal to distal airways [32,33],and distal airways have been shown to be less prone for goblet cell metaplasia by diminished expression of IL-13 signaling components, including IL-13 receptor IL-13Rα1, SPDEF and FOXA3 [34]. These considerations support our conclusion that patients with COPD have an increased expression of *SPDEF* and *MUC5AC* compared to controls and suggest that our results might even be an underestimation.

As expected we observed decreased expression of *FOXA2*, a known repressor of goblet cell differentiation [11,13,17,35], during IL-13-induced epithelial cell differentiation, accompanied by increased expression of *SPDEF*. As *FOXA2* is negatively regulated by SPDEF [9,36] and two putative binding sites for SPDEF locate in the promoter and first exon of *FOXA2*, SPDEF may down regulate *FOXA2* expression directly. *FOXA2* is also regulated by promoter DNA methylation. *FOXA2* was shown to be

repressed and hypermethylated in the promoter in lung cancer [25], whereas it was also shown that *FOXA2* was activated with hypermethyaltion in the promoter during endoderm development [37]. In our study, we observed that *FOXA2* was hypomethylated in cells from patients with COPD after goblet cell differentiation in the presence of IL-13 (CpG #14 and #15), but also in the absence of IL-13 (CpG #10, #11 and #15). In both cases, hypomethylation of *FOXA2* was not accompanied with a change in expression level. These phenomena could be explained by the minor methylation differences (around 2% to 4%), which might not result in any biological effect on transcription, or a decreased binding of repressive transcription factors/increased binding of active transcription factors.

It is of note that FOXA2 is also essential for proper establishment of cellular junctions and maintenance of polarity [37], whereas *FOXA2*, together with other apical junctional complex-related genes, was shown to be decreased (mRNA) in small airway epithelium of both healthy smokers and COPD smokers compared to non-smokers [19]. In agreement with previous findings, cultured bronchial epithelial cells from patients with COPD displayed abnormalities with reduced capacity to form epithelial junctions and regenerate a mucociliary epithelium [38,39] which might be driven by aberrant DNA methylation profiles. So it is important to validate our finding and further investigate the biological role of DNA methylation in *FOXA2* promoter during airway epithelial cell differentiation and also in COPD in the future.

We assessed DNA methylation in the total population of differentiated cells, of which the goblet cell population represents about 5%. Using pure cell populations of basal cells, ciliated cells, club (Clara) cells, and goblet cells in future studies will increase the opportunity to find more differentiated methylation loci/ region. However, even using the total cell population, our approach turned out to be sensitive enough to resolute the difference between COPD and control, which may be indicative for the magnitude of this methylation difference.

Altogether, we have demonstrated that *SPDEF* expression is increased during IL-13-induced goblet cell differentiation, which correlates to hypermethylation of CpG #8 in the *SPDEF* promoter. *SPDEF* expression is also higher in COPD-derived ALI cultures compared to control-derived ALI cultures in the absence of IL-13, which is accompanied with hypomethylation of CpG #6 in the *SPDEF* promoter. Moreover, *FOXA2* is hypomethylated (CpG #14 and #15) during IL-13-induced goblet cell differentiation and also hypomethylated (CpG #10 and #11) in COPD without a change in expression level. This shows the complex biology of airway epithelial cell differentiation where different transcription factors are involved and expression and DNA methylation mutual affect each other. Our study has shown the potential relevance of *SPDEF* regarding mucus hypersecretion in COPD and improved our insight, for development of epigenetic-based anti-mucus therapeutic strategies in the future.

Acknowledgements

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Conflict of interest: None declared.

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Supplementary Table S1 PCR and sequencing primers

Primer Name	Sequence (5'- 3')	Application
SPDEF Pyro-A F	GGGTTATGGGAGAGTAAGTTGT	PCR and sequencing for SPDEF-A pyrosequencing
SPDEF Pyro-A R	[Biotin]TCTATACCCCACAAAATCCTCAT	
SPDEF Pyro-A Seq	GTTGTTGGTTGGTTT	
SPDEF Pyro-B/C F	GGATTTTGTGGGGTATAGAGAA	PCR and sequencing for SPDEF-B/C pyrosequencing
SPDEF Pyro-B/C R	[Biotin]ATTACTACATAACCACTCAACTCATATT	
SPDEF Pyro-B Seq	GGGGTATAGAGAATATAGTT	
SPDEF Pyro-C Seq	TTTAGAATTTTAGTTTTGGATTTA	
SPDEF Pyro-D/E F	ATGAGTTGAGTGGTTATGTAGTAAT	PCR and sequencing for SPDEF-D/E pyrosequencing
SPDEF Pyro-D/E R	[Biotin]CCAACCCAAAACTACCTACTAAC	
SPDEF Pyro-D Seq	AGTGGTTATGTAGTAATTAATG	
SPDEF Pyro-E Seq	AATTAGGTTTTGGTTAATTT	
FOXA2 Pyro-F	GTGGGTATTTAGGTTGTGATTGAAAAG	PCR and sequencing for FOXA2 pyrosequencing
FOXA2 Pyro-R	ACCCCTCCCTATTACAATTCA	
FOXA2 Pyro Seq	GTTGTGATTGAAAAGTAATTTTG	

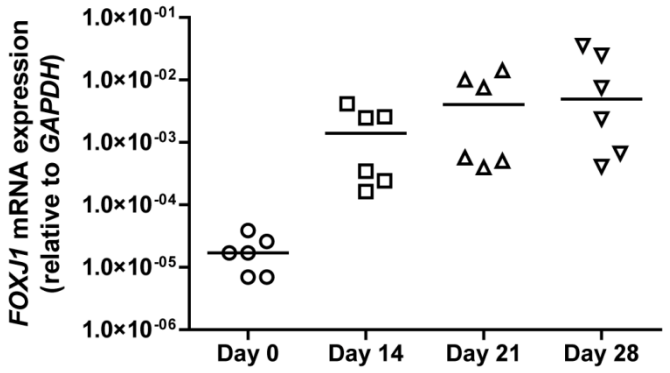

Figure S1 Characterization of the ciliated cell differentiation of the primary bronchial epithelial cells (PBEC) cultured in the air-liquid interface (ALI) model. *PBEC from control subjects 1-6 (Table 1, n=6) were cultured at ALI with IL-13*

stimulation and mRNA expression of FOXJ1 *was analyzed by real-time quantitative PCR at four different time points. Medians are indicated. Significance was tested by the Kruskal-Wallis nonparametric test with Dunn's posttest data. ns not significant, and **p<0.01; data from day 14, 21, and 28 were compared to day 0.*

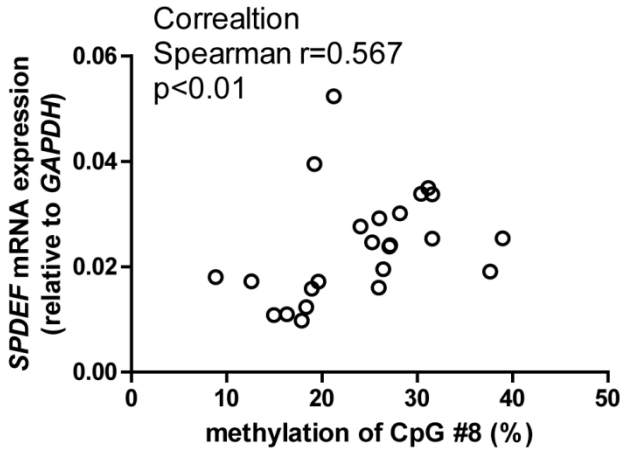

Figure S2 Correlation between methylation level of CpG #8 in the* SPDEF *promoter and* SPDEF *mRNA level during goblet cell differentiation of PBECs from control subjects. *Data represent as scatter plots on methylation level of CpG #8 (x-axis) and* SPDEF *mRNA level (y-axis) from the same sample. The correlation coefficient and significance were tested by Spearman nonparametric correlation test.*

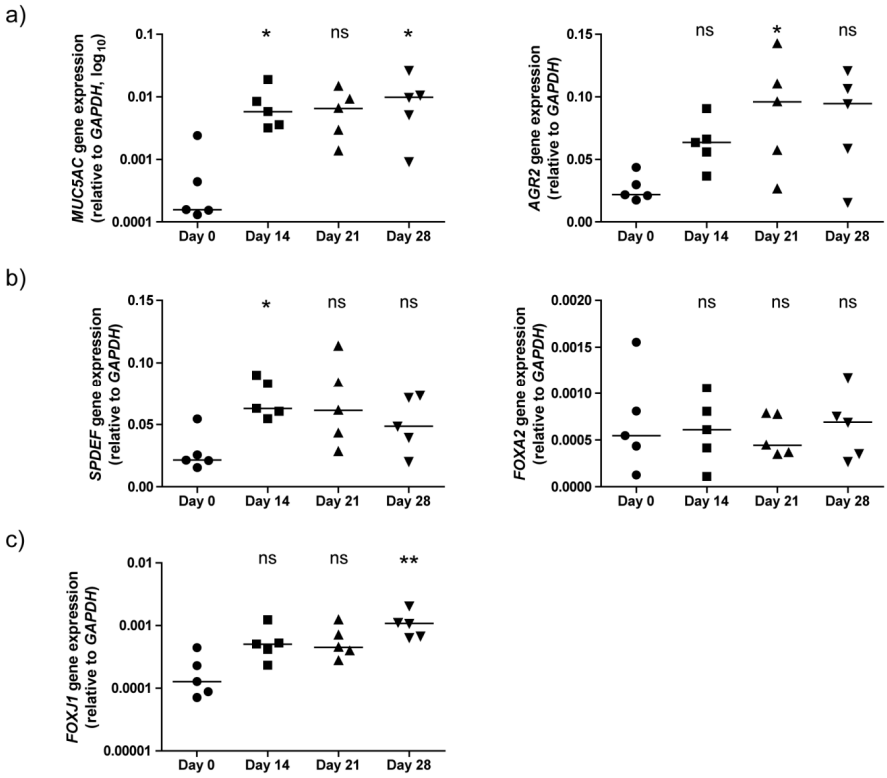

Figure S3 Characterization of the differentiation status of the PBEC from patients with COPD cultured in the air-liquid interface (ALI) model. *PBEC from patients with COPD1-5 (Table1, n=5) were cultured at ALI with IL-13 stimulation. mRNA expression of a)* MUC5AC *and* AGR2*, b)* SPDEF *and* FOXA2*, c)* FOXJ1 *were analyzed by real-time quantitative PCR at four different time points.*

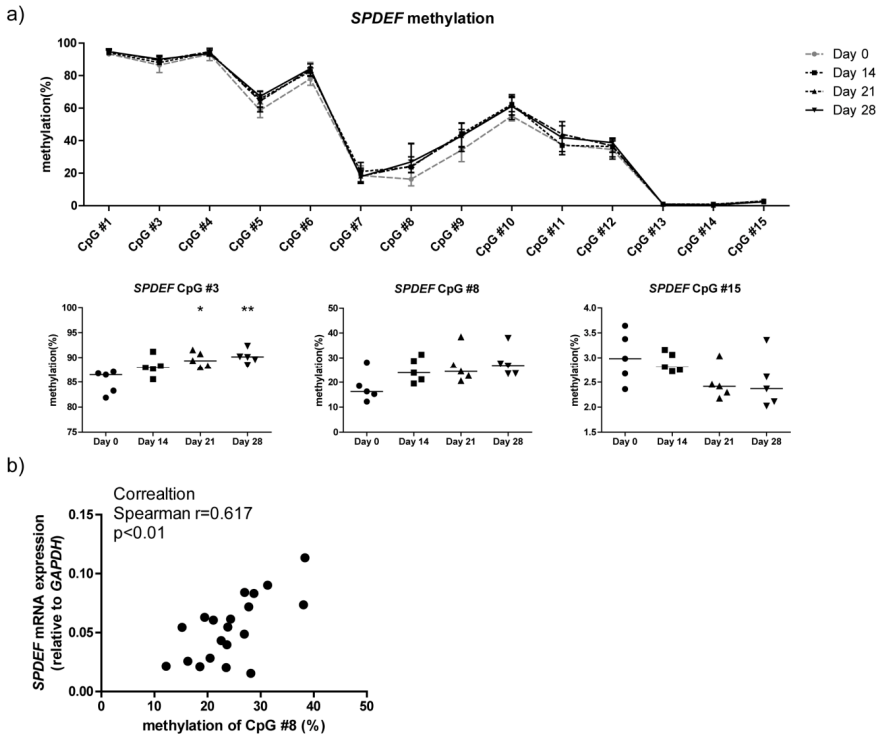

Figure S4 *Dynamic changes of DNA methylation in the* SPDEF *promoter during goblet cells differentiation of PBEC from patients with COPD. a) PBECs from patients with COPD 1-5 (Table 1, n=5) were cultured at ALI and DNA methylation levels were analysed at four different time points. Data represent the connected median methylation levels (Min to Max) of different CpG sites at each time points, and differential methylated CpG sites are specified with medians indicated. Significance was tested by the Kruskal-Wallis nonparametric test with Dunn's posttest data. ns not significant, *p<0.05, and **p<0.01; data from day 14, 21, and 28 were compared to day 0. b) Correlation between methylation level of CpG #8 in the* SPDEF *promoter and* SPDEF *mRNA level during goblet cells differentiation of PBECs from patients with COPD. Data represent as scatter plots on methylation level of CpG #8 (x-axis) and* SPDEF *mRNA level (y-axis) from the same sample. The correlation coefficient and significance were tested by Spearman nonparametric correlation test.*

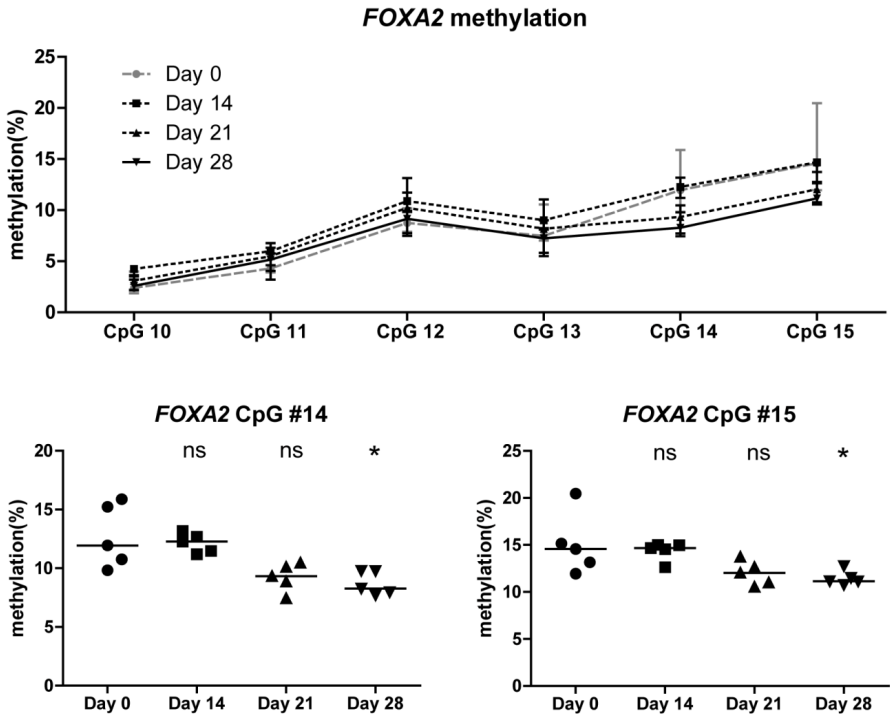

Figure S5 *Dynamic changes of DNA methylation in the* FOXA2 *promoter during goblet cells differentiation of PBEC from patients with COPD. a) PBECs from patients with COPD 1-5 (Table 1, n=5) were cultured at ALI and DNA methylation levels were analysed at four different time points. Data represent the connected median methylation levels (Min to Max) of different CpG sites at each time points, and differential methylated CpG sites are specified with medians indicated. Significance was tested by the Kruskal-Wallis nonparametric test with Dunn's posttest data. ns not significant, *p<0.05; data from day 14, 21, and 28 were compared to day 0.*

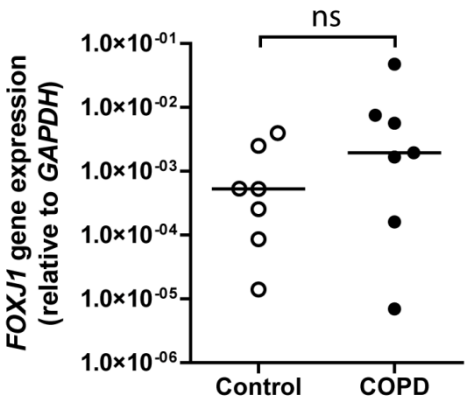

Figure S6 *Differential mRNA expression of* FOXJ1 *in the PBECs from COPD patients and control subjects. PBECs from control 7-17 and COPD 6-18 (Table 1) were differentiated at ALI for 14 days. Expression of* FOXJ1 *were analyzed by real-time quantitative PCR. Significance was tested by the Mann-Whitney U test. Medians are indicated. ns not significant.*

Chapter 5

Targeted epigenetic editing of SPDEF reduces mucus production in human airway epithelium

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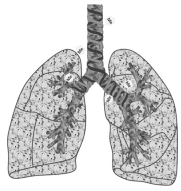

Abstract

Airway mucus hypersecretion contributes to the morbidity and mortality in patients with chronic inflammatory lung diseases. Reducing mucus production is crucial for improving patients' quality of life. The transcription factor SAM-pointed domain–containing Ets-like factor (*SPDEF*) plays a critical role in the regulation of mucus production, and therefore represents a potential therapeutic target. This study aims to reduce lung epithelial mucus production by targeted silencing *SPDEF* using the novel strategy epigenetic editing.

Here, four zinc finger proteins, engineered to target the *SPDEF* promoter, were fused to transcriptional repressor (KRAB), to DNA methyltransferase 3A (DNMT3A) or to histone methyltransferase G9a. Human airway epithelial A549 cells were transduced to express the fusion proteins.

We observed that all fusion proteins were able to effectively suppress both *SPDEF* mRNA and protein expression, and ZFs-DNMT3A induced *de novo* DNA methylation at the *SPDEF* promoter. Importantly, all editing approaches were accompanied by inhibition of downstream mucus-related genes Anterior gradient 2 (*AGR2*) and Mucin 5AC (*MUC5AC)* expression.

These results indicate efficient *SPDEF* silencing and down regulation of mucus related gene expression by epigenetic editing in human lung epithelial cells. This opens avenues for epigenetic editing as a novel therapeutic strategy to induce long-lasting mucus inhibition.

Keywords: *SPDEF*, epigenetic editing, mucus production, DNA methylation

Introduction

Airway epithelial mucus secretion and mucociliary clearance plays a key role in protective innate immune responses against inhaled noxious particles and microorganisms. However, excessive mucus production and secretion contributes to the pathogenesis of several chronic inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) (5, 7, 21). In patients with asthma and COPD, mucus hypersecretion is associated with cough and sputum production, respiratory infections, accelerated lung function decline, exacerbations and mortality (17, 26). Therefore, targeted treatment of pathologic airway mucus secretion is expected to not only improve symptoms of cough and dyspnea, but also decrease the frequency of disease-related exacerbations and decelerates the disease progression. In the past few years, in preclinical models relevant to COPD, several drugs were shown to reduce mucus hypersecretion (15). However, none of these drugs targeted the mucus producing cell itself.

Airway mucus contains mostly water and secreted mucins that contribute to the viscosity and elasticity of mucus gels. Mucin 5AC (*MUC5AC)* is the major secreted mucin, which is mainly produced by goblet cells in the airway epithelium. In chronic respiratory diseases, mucus hypersecretion is highly associated with increased numbers of goblet cells, as well as up regulated levels of mucin synthesis and secretion (5). SAM pointed domain-containing Ets transcription factor (*SPDEF*) has been reported to be a core transcription factor (TF) that, within a large network of genes, controls mucus production and secretion (2, 16, 27). In lung, *SPDEF* is selectively expressed in goblet cells lining the airways of patients with chronic lung disease (2) and mice exposed to allergens (19). In mice, the absence of *SPDEF* was shown to protect from goblet cell development after allergen exposure (2, 20). Moreover, knockdown of *SPDEF* with small interfering RNA (siRNA) was found to significantly reduce the expression of IL-13-induced *MUC5AC* expression and Anterior gradient 2 (*AGR2*) expression, which encodes a potential chaperone required for mucin packaging, in the human bronchial epithelial cell line 16HBE (28). These observations suggest

that *SPDEF* could be a potential therapeutic target of airway mucus hypersecretion. In this study we set out to silence *SPDEF* expression by epigenetic editing. Epigenetic editing is a novel approach to modulate epigenetic states locally by targeting an epigenetic enzyme to the locus of interest via DNA-targeting systems, such as zinc fingers (ZFs), transcription activator-like effectors (TALEs), or clustered regularly interspaced short palindromic repeats (CRISPRs) (4, 14). Compared to artificial transcription factors (ATFs), which exploit DNA-binding platforms to target transcriptional activators or repressors with no catalytic domain (such as super KRAB Domain, SKD), epigenetic editing has the promise to induce stable and inheritable gene modulation (22, 24). In this study, we provide proof-of-concept that *SPDEF* provides a promising target for epigenetic editing to prevent epithelial mucus production.

Materials and Methods

Cell culture

Human bronchial epithelial 16HBE 14o- (16HBE) and BEAS-2B, mucoepidermoid carcinoma NCI-H292 and type II alveolar carcinoma A549 cell lines were cultured as previously described (11). The human embryonic kidney HEK293T cell line were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (Biowhittaker, Verviers, Belgium). All culture media were supplemented with 2 mmol/L L-glutamine, 50 μg/mL gentamicin, and 10% FBS (Biowhittaker).

Plasmids Constructs

Four 18-bp zinc finger (ZF) protein target sites were selected within the *SPDEF* promoter using the website www.zincfingertools.org., as previously described (12). The target sequences are shown in Fig. 2a. The DNA sequences encoding the ZFs were synthesized by Bio Basic Canada. The

fragments encoding the ZFs were digested with BamHI/ NheI restriction enzymes (Thermo Fisher Scientific, Carlshad, USA) and cloned into a SKD-NLS-ZF-TRI FLAG backbone, which encodes SKD, a triple-FLAG tag and a nuclear localization signal (NLS) or a ZF- NLS-VP64-TRI FLAG backbone, which encodes a tetramer of Herpes Simplex Virus Viral Protein 16 (VP64). Then the SKD-NLS-ZF SPDEF-TRI FLAG fragments and the ZF SPDEF- NLS-VP64-TRI FLAG were XbaI/ NotI (Thermo Fisher Scientific) digested and subcloned into a dual promoter lentiviral vector pCDH-EF1-MCS-BGH PCK-GFP-T2A-Puro (SBI, Cat. #CD550A-1), obtaining constructs CD550A-1 SKD-ZF SPDEF and CD550A-1 ZF SPDEF-VP64. To obtain the constructs CD550A-1 ZF SPDEF-DNMT3A, the DNMT3A catalytic domain (kindly provided by Dr. A Jeltsch) was digested out from pMX-ZF-DNMT3A-IRES-GFP with AscI and PacI, to replace VP64 in the CD550A-1 ZF SPDEF-VP64 vector. Catalytically mutant of DNMT3A (E74A) (9) was generated by PCR-mediated site directed mutagenesis on CD550A-1 ZF SPDEF-DNMT3A. To obtain the constructs CD550A-1 ZF SPDEF-G9a and CD550A-1 ZF SPDEF-G9a W1050A, the G9a catalytic domain and its mutant was digested out from pMX-E2C-G9a and pMX-E2C-G9a W1050A (6) with AscI and PacI, to replace VP64 in the CD550A-1 ZF SPDEF-VP64. To construct the CD550A-1 ZF SPDEF without effector domains (SPDEF-NOED), VP64 in the CD550A-1 ZF SPDEF-VP64 was swapped out with PCR by a multiple cloning site, including restriction sites for AscI, NsiI, BclI, SwaI, and PacI. The primer information is presented in Table 1. We verified all constructs by DNA Sanger sequencing (Baseclear, Leiden, the Netherlands).

Lentiviral transduction

The lentiviral CD550A-1 constructs, encoding the *SPDEF* targeted ATFs and epigenetic editors, were co-transfected with the third generation packaging plasmids pMDLg/pRRE, pRSV-Rev, pMSV-VSVG into HEK293T cells using the calcium phosphate transfection method to produce lentiviral particles. The supernatant of HEK293T cells containing virus was harvested at 48 and 72

hours after transfection. Host A549 cells were seeded in six-well plates with a density of 80,000 cells per well and transduced on two consecutive days with the viral supernatant, supplemented with 8 µg/mL polybrene (Sigma-Alrich, Zwijndrecht, Netherlands). The positive transduced cells were selected in 8 µg/mL puromycin supplemented medium for four days from 72h after the last transduction and then were cultured in 1 µg/mL puromycin supplemented medium. Medium was refreshed every 2-3 days. Ten days after the last transduction, cells were harvested for western blot, as well as RNA and DNA extraction. In the meantime, cells were grown on coverslips for immunocytochemistry (IHC) and harvested for chromatin immunoprecipitation.

Detection of mRNA expression by quantitative real-time PCR

Total RNA was extracted from A549 cells using Trizol reagent (Thermo Fisher Scientific) and 500 ng was used for cDNA synthesis with random primers using Superscript II RNase H - Reverse transcriptase (Thermo Fisher Scientific). *SPDEF*, *MUC5AC*, *AGR2* and *GAPDH* expression was quantified using qPCR MasterMix Plus (Eurogentec, Belgium) and Taqman gene expression assays (*SPDEF*: Hs01026050_m1; *MUC5AC*: Hs00873651_Mh; *AGR2*: Hs00356521_m1; *GAPDH*: Hs02758991_g1, Thermo Fisher Scientific), mRNA expression of the fusion proteins (FLAG tag) using SYBR® Green PCR Master Mix (Thermo Fisher Scientific) and gene-specific primers (Table 1) with the LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). Data were analyzed with LightCycler® 480 SW 1.5 software (Roche) and the Fit points method, according to the manufacturer's instructions. Expression levels relative to *GAPDH* were determined with the formula $2^{-\Delta Cp}$ (Cp means crossing points).

Methylation analysis by pyrosequencing

For DNA methylation analysis of the target regions, genomic DNA was extracted with chloroform-isopropanol and was bisulfite converted using the EZ DNA Methylation-Kit (Zymo Research), following the manufacturer's protocol. Bisulfite-converted DNA was analyzed by pyrosequencing as previously described (3). The primer information for pyrosequencing is presented in Table 1.

Histone modification analysis by Chromatin immunoprecipitation and qPCR

Histone modification induced by ZFs-G9a was analyzed by ChIP as previously described (8). Briefly, A549 cells were fixed with 1% formaldehyde at 37 °C for 10 min and subsequently lysed and sonicated using a Bioruptor (Diagenode; High, 30 sec on, 30 sec off, total time 15 minutes). Sheared chromatin was cleared by centrifuge at 4°C (12,000 × g, 10 minutes). Four microgram of specific antibodies [normal rabbit IgG (abcam, ab46540), H3K9me2 (Milipore, 07-441)] were bound to 50 µl of magnetic Dynabeads (Thermo Fisher Scientific) during 15 minutes incubation, then unbound antibodies were washed-off. Sheared chromatin 0.25 million cells was added to the antibody precoated magnetic Dynabeads (rotating overnight at 4°C). Next day, the magnetic Dynabeads were washed three times with PBS, and chromatin was eluted with 1% (w/v) SDS and 100 mmol/L NaHCO3. Subsequently, the elutes were treated with RNase (Roche) for four hours and proteinase K (Roche) for one hour at 62°C. Then, the purified DNA by column purification (Qiagen) could be analyzed with quantitative PCR (qPCR).

To assess the induction of histone marks and their spreading, several primer pairs were used for the SPDEF promoter (Table 1). qPCR was conducted using SYBR Green PCR Master Mix (Thermo Fisher Scientific) on an LightCycler® 480 Real-Time PCR System (Roche). To calculate the fold

induction/reduction of histone marks we used the formula: Percentage input = $2^{(Cpinput-CpChIP)}$ dilution × factor × 100.

Detection of protein expression by western blot

Transduced A549 cells were lysed in RIPA buffer and proteins were analyzed by standard western blotting as previously described (3). Then, the blots were incubated with an rabbit anti-human SPDEF antibody (Santa Cruz, sc-67022), mouse anti-FLAG (Sigma, F3165) and mouse anti-GAPDH (Santa Cruz, sc-47724) at 4°C, overnight, followed by incubation with an horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit and rabbit anti-mouse antibody (Dako, Glostrup, Denmark). Protein expression was visualized using the Pierce ECL2 chemoluminescence detection kit (Thermo Fisher Scientific) and Gel Doc™ XR+ imaging systems (Bio-Rad Laboratories). Data were analyzed with Gel Doc™ XR+ Image Lab™ Software.

Detection of MUC5ACc protein expression by immunocytochemistry staining

Cells, grown on coverslips (Menzel-Gläser, 12 mm in diameter), were washed with PBS and fixed with 2% (w/v) Paraformaldehyde for 20 min. Cells were stained with primary antibody against MUC5AC (abcam, ab3649), followed by HRP-conjugated secondary antibody. The peroxidase was visualized by staining with AEC (3-amino-9 ethylcarbazole), followed by hematoxylin counterstaining. The cover glasses were mounted with Kaiser's glycerol-gelatin (37°C) and scanned into digital whole slides images using the NanoZoomer series scanning devices. The assessment of immunochemistry staining intensity was performed semiquantitatively in a blinded fashion. MUC5AC staining cells were categorized as follows: negative, weak-positive and strong-positive.

Statistics

All transduction experiments were performed three times independently. Data were analyzed using Student's t-test (one-tailed). Data were considered to be statistically significant if $P<0.05$. Data were expressed as mean ± SEM and calculated using Prism v5.0 (GraphPad software).

Table 1 PCR and sequencing primers

Primer Name	Sequence (5'- 3')	Application
SPDEF Pyro-A F	GGGTTATGGGAGAGTAAGTTGT	PCR and sequencing for SPDEF-A pyrosequencing
SPDEF Pyro-A R	[Biotin]TCTATACCCCACAAAATCCTCAT	
SPDEF Pyro-A Seq	GTTGTTGGTTGGTTT	
SPDEF Pyro-B/C F	GGATTTTGTGGGGTATAGAGAA	PCR and sequencing for SPDEF-B/C pyrosequencing
SPDEF Pyro-B/C R	[Biotin]ATTACTACATAACCACTCAACTCATATT	
SPDEF Pyro-B Seq	GGGGTATAGAGAATATAGTT	
SPDEF Pyro-C Seq	TTTAGAATTTTAGTTTTGGATTTA	
SPDEF Pyro-D/E F	ATGAGTTGAGTGGTTATGTAGTAAT	PCR and sequencing for SPDEF-D/E pyrosequencing
SPDEF Pyro-D/E R	[Biotin]CCAACCCAAAACTACCTACTAAC	
SPDEF Pyro-D Seq	AGTGGTTATGTAGTAATTAATG	
SPDEF Pyro-E Seq	AATTAGGTTTTGGTTAATTT	
DNMT3a-E74A F	CATTGCCTCCGCCGTGTGTGAGG	PCR for DNMT3a-E74A site mutagenesis
DNMT3a-E74A R	TAGCGGTCCACTTGGATGC	
NOED F	CGCGCCATGCATGATCATTTAAATTTAAT	PCR for NOED cloning
NOED R	TAAATTTAAATGATCATGCATGG	
SPDEF-ChIP-region 1 F	GCATGGGTGGTTCTGGATCT	ChIP-qRT-PCR for SPDEF region 1
SPDEF-ChIP-region 1 R	GCCAGAGATACGTCGAGTGG	
SPDEF-ChIP-region 2 F	GCAGCAACCAATGAACGAGTG	ChIP-qRT-PCR for SPDEF region 2
SPDEF-ChIP-region 2 R	ATTAACCCTTGCAGGTCTCCC	
SPDEF-ChIP-region 3 F	CCAGCACATTCCTGCACTCT	ChIP-qRT-PCR for SPDEF region 3
SPDEF-ChIP-region 3 R	CAACCTGAGGGGCTTGCAG	
FLAG-F	TGAATCGGTAGGAATTCGCGG	qRT-PCR for *FLAG*
FLAG-R	GGGAGGGGCAAACAACAGAT	
GAPDH-F	CCACATCGCTCAGACACCAT	qRT-PCR for *GAPDH*
GAPDH-R	GCGCCCAATACGACCAAAT	

Results

SPDEF down regulation by ATFs and subsequent repression of mucus-related genes

To select a suitable model to study *SPDEF* down regulation, *SPDEF* expression was determined in four different human epithelial cell lines: A549, H292, BEAS-2B and 16HBE. A549 cells demonstrated the highest expression of *SPDEF*, both at mRNA level (Fig. 1A) and at protein level (Fig. 1B). The high expression of *SPDEF* in A549 and H292 cells was accompanied by a low degree of DNA methylation at the CpG sites surrounding the transcription start site (TSS) (A549: CpG sites #13: 2.7%, CpG sites #14: 4.6%, CpG sites #15: 3.1%; H292: CpG sites #13: 1.9%, CpG sites #14: 4.2%, CpG sites #15: 3.2%), whereas the undetectable transcription levels of *SPDEF* in BEAS-2B and 16HBE were accompanied by a high level of DNA methylation (BEAS-2B: CpG sites #13: 34.9%, CpG sites #14: 40.6%, CpG sites #15: 26.4%; 16HBE: CpG sites #13: 75.9%, CpG sites #14: 68.5%, CpG sites #15: 41.0%) (Fig. 1D). Differential expression of *MUC5AC* was consistent with the observed *SPDEF* expression, with the highest *MUC5AC* expression in A549 cells (Fig. 1C). To explore effective *SPDEF* down regulation, we chose the highest *SPDEF* and *MUC5AC* expressing cell line (A549) as a model.

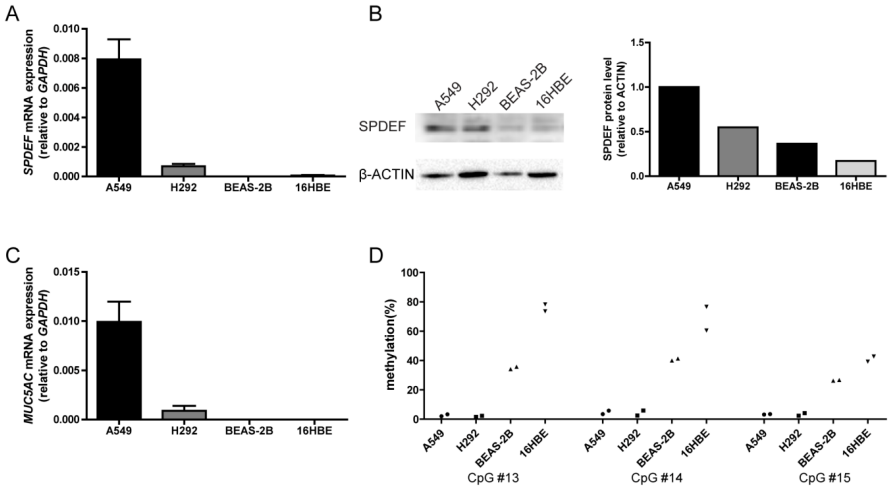

Figure 1 Expression of SPDEF (mRNA and protein) is associated with DNA methylation and* MUC5AC *expression. *Quantification of the mRNA levels of* SPDEF *(A) and* MUC5AC *(C) in a panel of human epithelial cell lines (A549, H292, BEAS-2B, and 16HBE) by qRT-PCR. Bars represent the average (±SEM) of three independent experiments. (B) Representative visualization of SPDEF protein expression (left) and quantification relative to β-ACTIN (right), as conducted by western blot. An anti-β-ACTIN antibody was used as a loading control. (D) Quantitative analysis of the methylation levels of three CpG sites surrounding transcription start site (TSS) by pyrosequencing. Scatter plots show two independent experiments.*

In order to down regulate *SPDEF* expression, four ZFs were designed to bind 18-base pair regions in the *SPDEF* promoter (SPDEF1, SPDEF2, SPDEF3, SPDEF4) and were sub-cloned into lentiviral constructs containing SKD (Fig. 2A). A549 cells were transduced to express the ATF using these lentiviral constructs. To enrich for cells expressing the ATF, the lentiviral transduced cells were positively selected based on puromycin resistance. Correct size of ATFs was confirmed by western blot (Fig. 2C) and their nuclear location by immunochemistry staining (Fig. 3C).

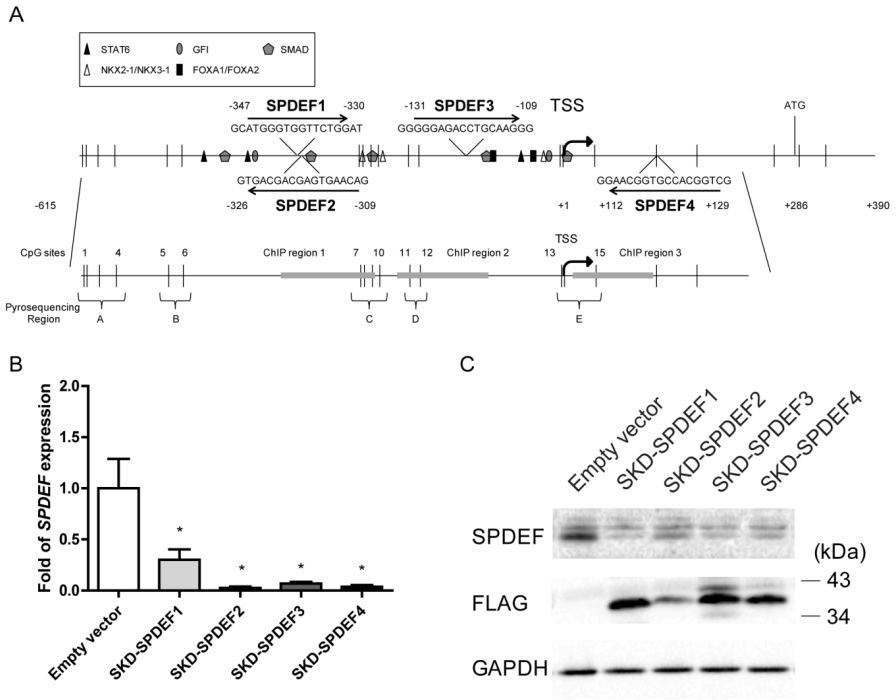

Figure 2* SPDEF-*targeted silencing by ATFs in A549 cells. *(A) Schematic representations of the promoter region of the* SPDEF *gene, outlining the putative binding sites for transcription factors (STAT6, NKX2-1/NKX3-1, GFI, FOXA2/FOXA1, SMAD) (MatInspector) and the target sequences of zinc fingers: SPDEF1, SPDEF2, SPDEF3, and SPDEF4. Arrows show the orientation of the 18-bp binding site in the promoter. Location of ZF was shown relative to the TSS (+1). The translation start site was shown as ATG (+286). CpGs are indicated as vertical bars. DNA methylation status of 15 CpGs was analyzed using pyrosequencing for the indicated areas. Histone modification of H3K9me2 was assessed for the ChIP regions (gray boxs). (B) Relative* SPDEF *mRNA expression, normalized to the empty vector, assessed by qRT-PCR in transduced A549 cells. Data are presented as mean (±SEM) of three independent experiments. Statistical significance was analyzed using t test (*P<0.05). (C) SPDEF protein expression in transduced A549 cells, as conducted by western blot. An anti- Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used as a loading control. An anti-FLAG antibody was used to detect the ATFs, which were designed with a C-terminal 3×FLAG tag. Blot pictures shown are representative of two independent experiments.*

Next, we examined the ability of the four ATFs to down regulate *SPDEF* mRNA expression in A549 cells. As shown in Fig. 2B, all four ATFs significantly down regulated *SPDEF* expression, demonstrating 70, 97, 93, and 96% respectively down regulation relative to empty vector control, which was confirmed at the protein level (Fig. 2C).

As *SPDEF* regulates a network of genes associated with mucus production (2, 20, 27), we investigated whether the down regulation of *SPDEF* expression mediated by ATFs indeed results in reduced mucus production. Therefore, the expression level of two downstream mucus-related genes was investigated in the ATF-expressing A549 cells. We found that expression of *AGR2* was significantly down regulated by SKD-SPDEF2 (90.9%±35.4% repression), SKD-SPDEF3 (79.3%±35.9% repression) and SKD-SPDEF4 (86.2%±35.4% repression) (Fig. 3A). *MUC5AC* was consistently, yet not significantly, down regulated in response to *SPDEF* repression (Fig. 3B). However, MUC5AC immunochemistry staining on ATF-transduced A549 cells supports successful inhibition at the protein level (Fig. 3C and 3D).

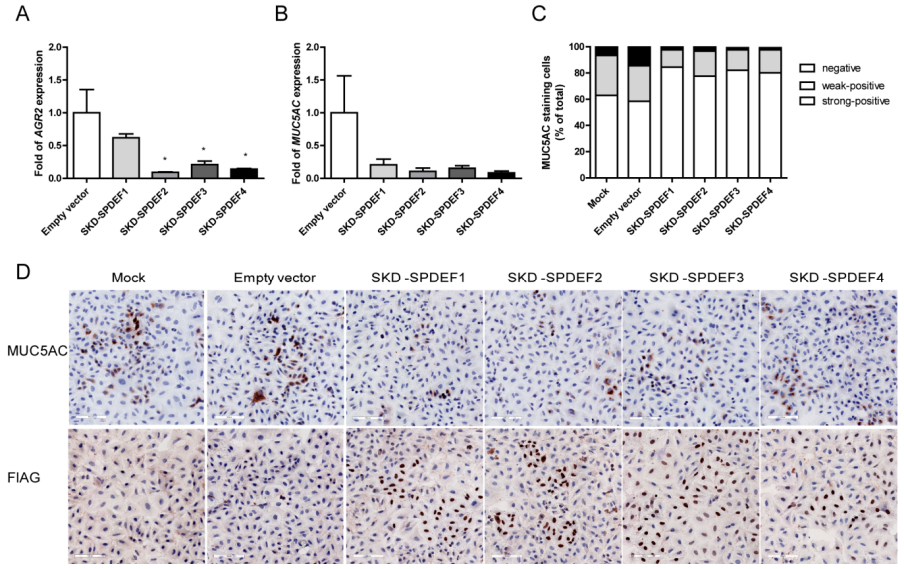

***Figure 3 Changes in downstream mucus-related genes after ATFs induced silencing of* SPDEF.** *(A)* MUC5AC *and (B)* AGR2 *mRNA expression were investigated by quantitative RT-PCR. (C)Quantification of MUC5AC negative, weak- and strong-positive A549 cells after ATF treatment. Counting of cells was performed in a blinded fashion. Solid bars, strong positive; shaded bars, weak positive; open bars, negative. Results represent the average of two independent experiments. (D) Representative photographs (original magnification, ×200) from immunocytochemistry staining for MUC5AC in ATFs treated A549 cells. Red-stained cells are MUC5AC-positive. Nuclei were counterstained with hematoxylin. Scale bar: 100 µm.*

SPDEF silencing by targeted epigenetic editing

In order to achieve the stable gene silencing, we set out to direct DNA methylation onto the *SPDEF* promoter. As DNA methylation levels of CpG sites #13 (-3), #14 (-1) and #15 (+40) around the TSS negatively correlated with *SPDEF* expression, ZF SPDEF3 targeting location -131 to -114 was coupled to the catalytic domain of DNMT3A. To investigate the induced DNA methylation in the promoter region of SPDEF, 15 CpG sites were screened with pyrosequencing (Fig. 4). We found that DNA methylation was

induced on CpGs sites #14 and 15, and not on CpG sites #1-13. In further experiments, CpG sites #13-15 were analyzed. SPDEF3-DNMT3A consistently deposited DNA methylation onto two CpG sites (CpG #14: 6.6 ± 0.8%; CpG #15: 10.5 ± 1.3%), compared with SPDEF3-NOED (CpG #site 14: 3.9 ± 0.3%; CpG #15: 5.2 ± 0.8%) (Fig. 5B). To determine whether the observed increase in DNA methylation was caused by the catalytic activity of the DNMT3A enzyme, a catalytic mutant of DNMT3A (DNMT3A E74A) was constructed and used in a separate set of experiments. No increase in DNA methylation was observed for CpG sites #13-15 in SPDEF3-DNMT3A E74A treated cells (Fig. 5C). To investigate whether the ZF directed DNMT3A was able to reduce target gene transcription, *SPDEF* mRNA expression was investigated (Fig. 7A, left panel). SPDEF3-DNMT3A was able to down regulate *SPDEF* expression (73.7%±29.6% repression), which was equally efficient as repression induced by the positive control SKD-SPDEF3 (77.1%±25.7% repression). Interestingly, the construct that lacked the effector domain, SPDEF3-NOED, also reduced *SPDEF* expression significantly (74.7%±26.2% repression). Upon ZFs fused with the histone methyltransferase G9A, no further decrease of *SPDEF* expression was observed compared to SPDEF3-NOED (Fig. 7A, right panel), and no H3K9me2 marks were detected in the examined region (Fig. 6). To determine the influence of location, another ZF (SPDEF4: target sequence +112 to +129) was tested to target DNMT3A or G9A to the *SPDEF* promoter (Fig. 5A and 6A). Again, fusion of either epigenetic editor did not hamper the repressive activity of ZF SPDEF4 itself (Fig. 7A). The expression of the fusion proteins was confirmed by the mRNA expression the FLAG-tag (Fig. 8).

Down regulation of *SPDEF* by SPDEF3-DNMT3A, SPDEF4-DNMT3A and SPDEF4-G9A was confirmed at the protein level by western blot (Fig. 9). Importantly, expression of downstream mucus related genes *AGR2* and *MUC5AC* was also down regulated by these constructs (Fig. 7B and 7C).

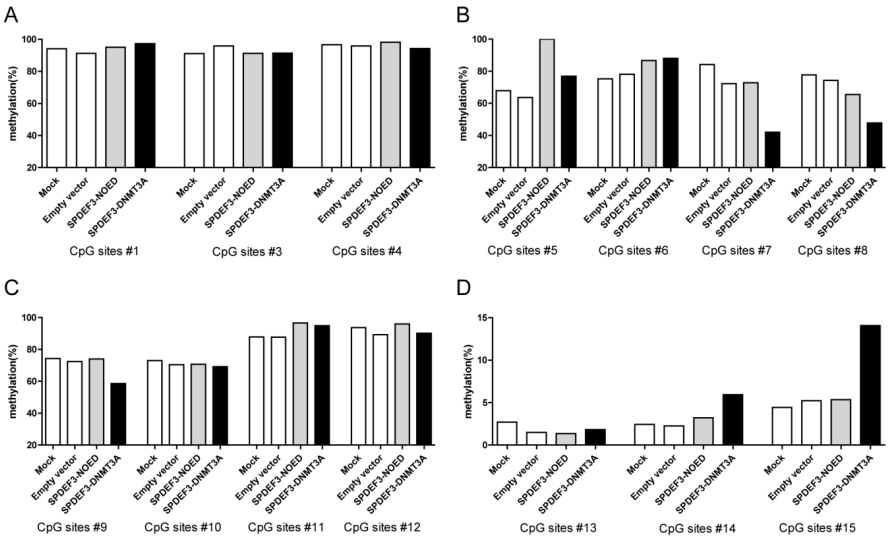

Figure 4 Screening of the DNA methylation changes after targeting DNMT3A to* SPDEF *promoter. *Quantitative analysis the percentage of methylation for 14 CpG sites in* SPDEF *promoter by pyrosequencing in A549 cells treated with mock, empty vector, SPDEF3-NOED and SPDEF3-DNMT3A in one experiment. (A) CpG sites #1, #3, and #4; (B) CpG sites #5-8; (C) CpG sites #9-12; (D) CpG sites #13-15.*

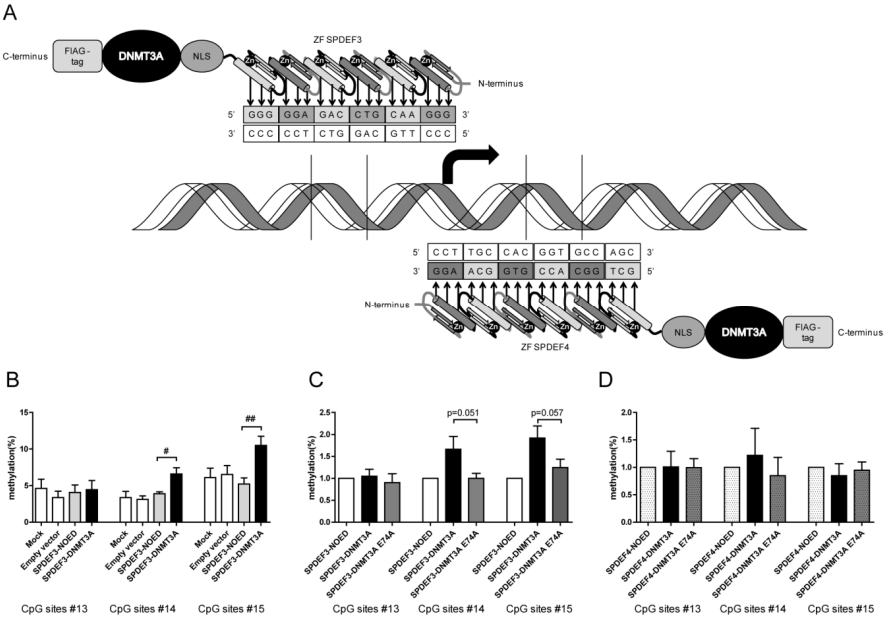

Figure 5 DNA methylation changes after targeting DNMT3A to* SPDEF *promoter. *(A) Schematic presentation of SPDEF3-DNMT3A and SPDEF4-DNMT3A, and their binding location relative to TSS. (B) Quantitative analysis the percentage of methylation for target CpG sites (#13, #14 and #15) by pyrosequencing in A549 cells treated with mock, empty vector, SPDEF3-NOED and SPDEF3-DNMT3A. (C) Relative DNA methylation level of A549 cells after treatment with SPDEF3-NOED, SPDEF3-DNMT3A and SPDEF3-DNMT3A E74A normalized to SPDEF3-NOED. (D) Relative DNA methylation level of A549 cells after treatment with SPDEF4-NOED, SPDEF4-DNMT3A and SPDEF4-DNMT3A E74A normalized to SPDEF4-NOED. All bars represent the mean of at least three independent experiments ±SEM. Statistical significance was analyzed using t test (#P<0.05, ##P<0.01, compared between two indicated columns).*

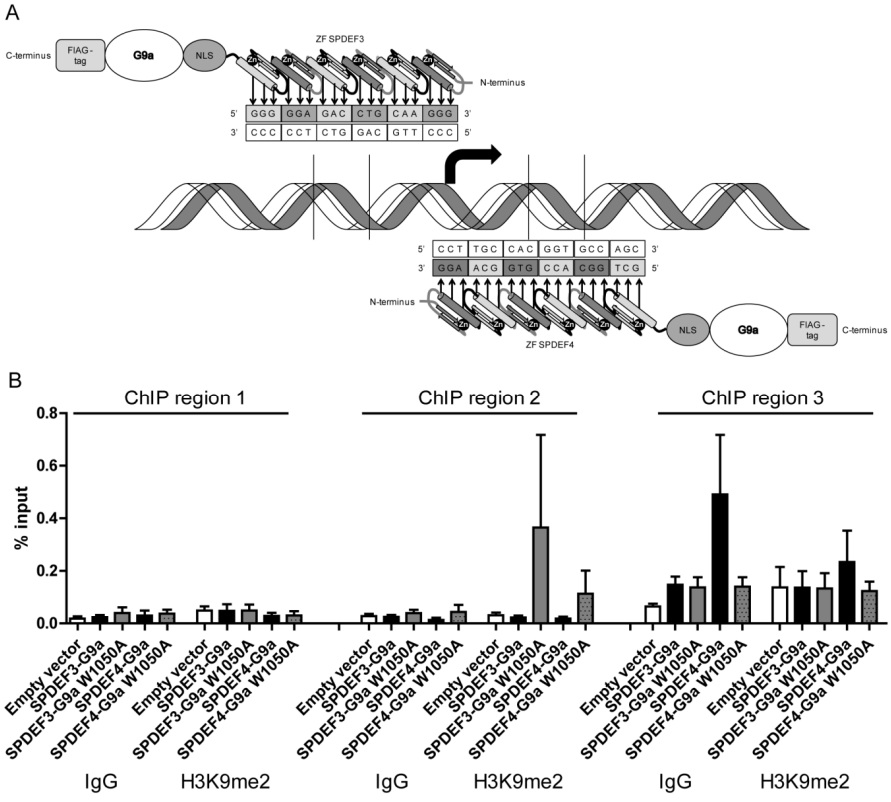

Figure 6 Changes in histone mark H3K9me2 after targeting G9a to* SPDEF *promoter. *(A) Schematic presentation of SPDEF3-G9a and SPDEF4-G9a, and their binding location relative to TSS. (B) Induction of H3K9me2 was assessed by quantitative ChIP for three regions of the* SPDEF *promoter in the transduced A549 cells. Data are presented as percentage of input. The bars represent the average*

(±SEM) of three independent experiments. IgG was used as negative control for immunoprecipitation.

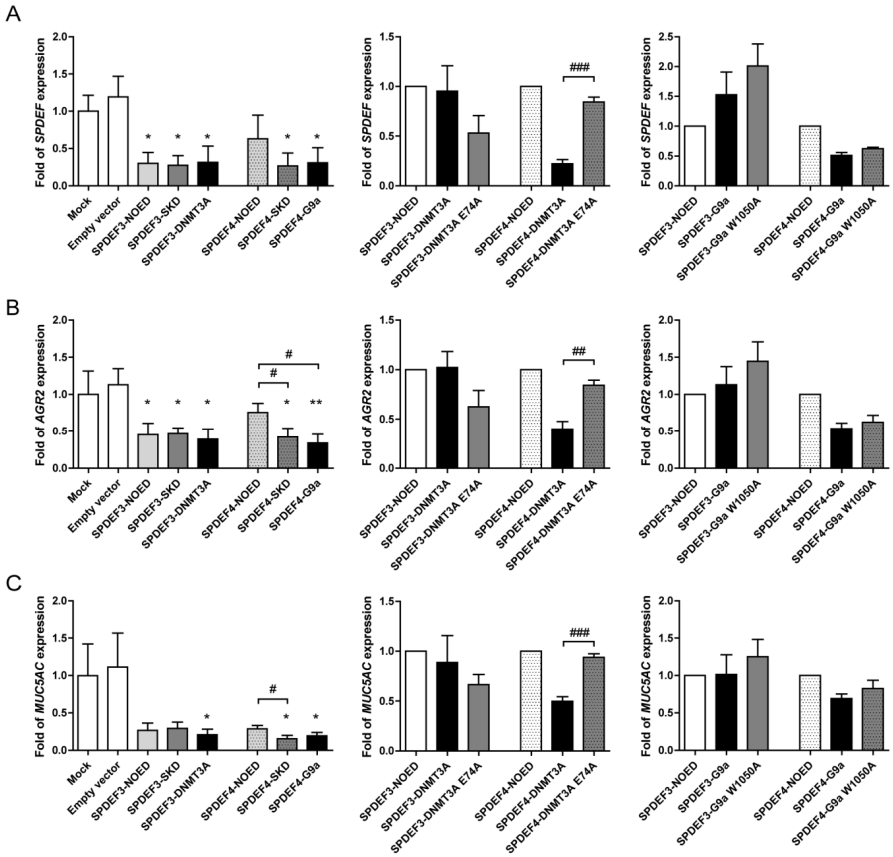

Figure 7* SPDEF *and downstream mucus related genes expression changes after targeting DNMT3A and G9a to* SPDEF *promoter. *A549 cells were treated with ZFs fused with different effector domains (SKD, DNMT3A, G9a, and the respective mutants DNMT3A E74A and G9a W1050A). mRNA level of (A)* SPDEF*, (B)* AGR2 *and (C)* MUC5AC *were determined by quantitative RT-PCR on treated A549 cells. The expression of* SPDEF *was relative to* GAPDH *and normalized to mock treated cells (left panel), or normalized to ZF-NOED (middle and right panels). The bars represent the mean of three independent experiments ±SEM. Statistical significance was analyzed using t test (*P<0.05, **P<0.01, ***P<0.001, compared to empty vector; #P<0.05, ##P<0.01, ###P<0.001, compared between two indicated columns).*

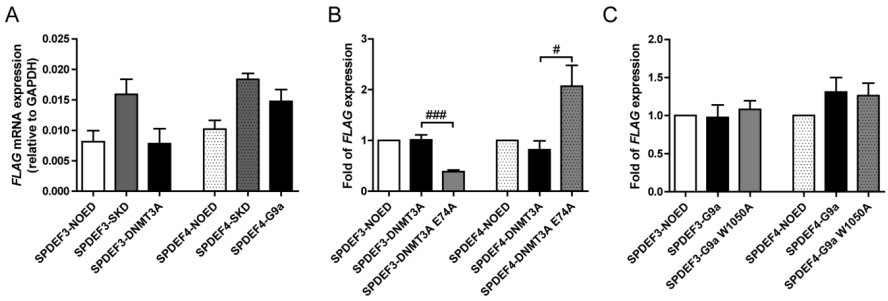

Figure 8 Expression of ZF-ED after A549 cells treated with ZF fused to different effector domain (SKD, DNMT3A, G9a, and respective mutant DNMT3A E74A and G9a W1050A). *The expression of ZF-ED was represented as the FLAG-tag expression relative to* GAPDH *(A), and normalized to ZF-NOED (B and C). The bars represent the mean of three independent experiments ±SEM. Statistical significance was analyzed using t test (#P<0.05, ###P<0.001, compared between two indicated columns).*

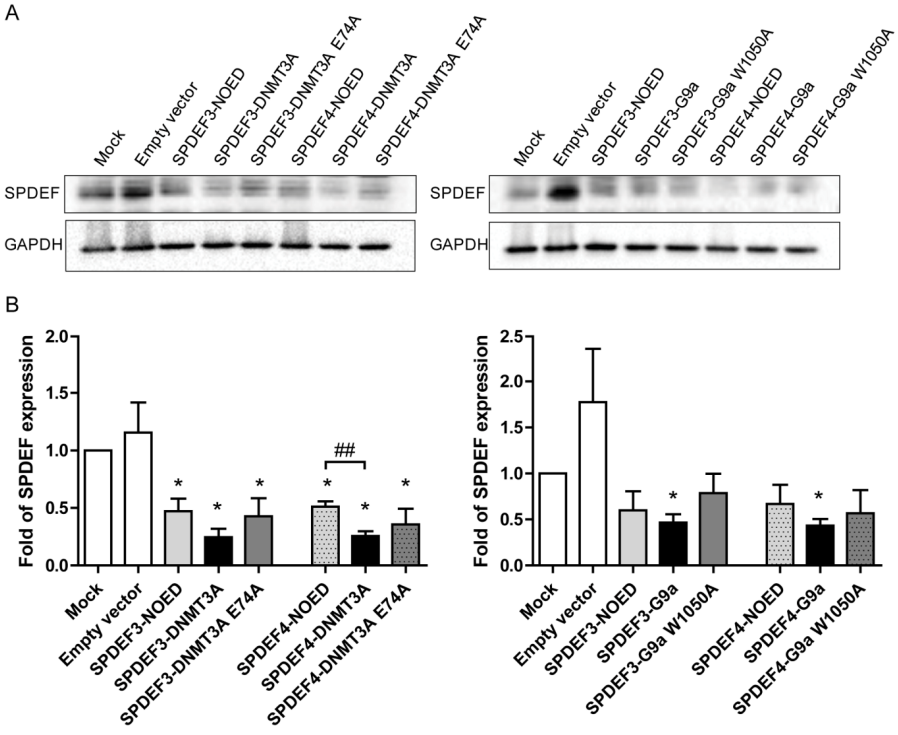

Figure 9 Quantification of the changes of SPDEF protein levels in A549 cells treated with* SPDEF *targeted DNMT3A and G9a. *A549 cells were treated with ZF fused with different effector domains (SKD, DNMT3A, G9a, and respective mutant*

*DNMT3A E74A and G9a W1050A). (A) Protein expression of SPDEF was assessed by Western blot. An anti-GAPDH antibody was used as a loading control. Blot pictures shown are representative of three independent experiments. (B) Densitometric values of SPDEF were normalized against the loading control, GAPDH. The relative level (% of mock) of SPDEF was shown with the average of three independent experiments ±SEM. Statistical significance was analyzed using t test (*P<0.05, **P<0.01, compared to empty vector; ##P<0.01, compared between two indicated columns).*

Lower number of MUC5AC positive cells after targeted silencing *SPDEF* by epigenetic editing

The effect of *SPDEF* inhibition on mucus production was determined by quantification of the number of MUC5AC positive cells. Transduced A549 cells were seeded on cover slips and examined by immunochemistry staining. Cells treated with SPDEF3-DNMT3A had significantly lower numbers of MUC5AC positive cells compared to empty vector treated cells (Fig. 10A), as expected from the mRNA data. Interestingly, *SPDEF* silencing was most effective within the MUC5AC strong positive cell population. Within this population, both SPDEF3-DNMT3A and SPDEF4-G9a treatment resulted in lower numbers of MUC5AC strong positive (Fig. 10B).

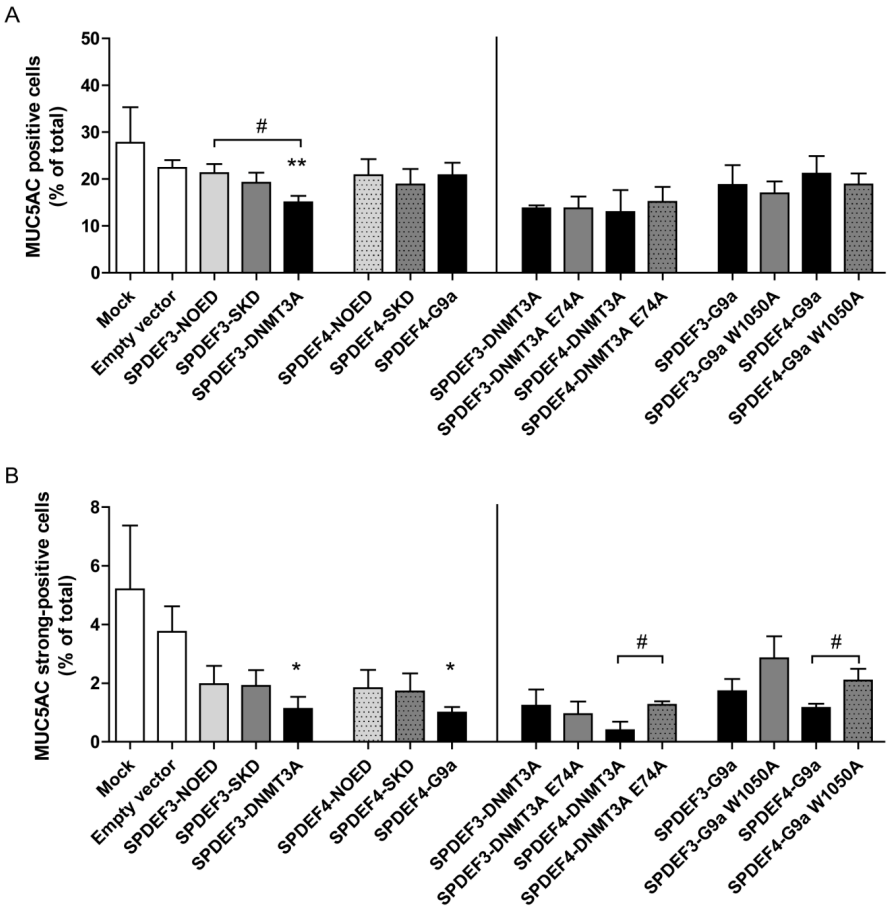

Figure 10 Quantification of MUC5AC positive A549 cells after treatment with SPDEF targeted DNMT3A and G9a. A549 cells were treated with ZFs fused with different effector domains (SKD, DNMT3A, G9a, and respective mutant DNMT3A E74A and G9a W1050A) and grown on coverslips. *Immunocytochemistry staining for MUC5AC was quantified to negative, weak-positive and strong-positive in a blinded fashion. (A) Percentage of MUC5AC positive cells in the total cell populations. (B) Percentage of MUC5AC strong-positive cells in the total cell populations. Results are represented as average (±SEM) of three independent experiments. Statistical significance was analyzed using t test (*P<0.05, **P<0.01, compared to empty vector; #P<0.05, compared between two indicated columns).*

Discussion

Based on its important role in goblet cell differentiation and mucus production (2, 20), we reasoned that *SPDEF* could be a suitable therapeutic target against mucus hypersecretion. In this study, we were able to silence *SPDEF* expression in the human alveolar epithelial cell line A549, using a novel strategy: engineered *SPDEF* targeting ZF proteins directing transcriptional repressor (SKD) as well as epigenetic enzymes (DNMT3A and G9A). The repression of *SPDEF* was accompanied by lower expression of mucus-related genes *MUC5AC* and *AGR2*, as well as lower numbers of MUC5AC positive cells.

Our data provides an original proof-of-concept study supporting *SPDEF* as a promising therapeutic target for inhibiting mucus production. As previously reported, knockdown of *SPDEF* using siRNA was able to reduce the IL-13-induced expression of MUC5AC and AGR2 in human airway epithelial 16HBE cells (28). The principle of siRNA is to target and degrade mRNA. Because of the constant production of mRNA, the silencing effect of siRNA is generally transient and it has to be delivered repeatedly in clinical application. Epigenetic editing would be a superior strategy because the effect would be sustained after clearance of the drug (hit and run approach) (4). In order to down-regulate SPDEF expression directly at the transcriptional level, four sequence-specific ZFs were generated. ZFs were first linked to SKD to test the functionality of the DNA binding domain because SKD can cause transient gene silencing by indirectly recruiting chromatin remodelers and histone-modifying enzymes (22, 25). These four ATFs (ZF-SKD) strongly reduced *SPDEF* expression and nearly abolished all expression of *SPDEF* in A549 cells. More importantly, *SPDEF* silencing resulted in the additional down regulation of MUC5AC mRNA and protein expression as well, indicating successful inhibition of mucin synthesis.

Next, ZFs were fused to catalytic domains of epigenetic enzymes (DNMT3A and G9A), aiming for longer term gene silencing by changing the epigenetic state of the targeted gene. ZF-targeted DNA methylation was recently successfully used for silencing several cancer-associated genes, including

VEGF-A, SOXA2, and EpCAM (18, 22-24). Here, we took advantage of this approach by using two different ZFs engineered close to the TSS (SPDEF3 and SPDEF4), to down regulate *SPDEF* expression. In this area, high expression of *SPDEF* was accompanied by lower DNA methylation of CpG sites, particularly those surrounding the TSS, where DNA methylation is tightly linked to transcriptional silencing (1). The occlusion binding of TF also explains our observation that ZFs without effector domains effectively silenced *SPDEF* expression. However, as the DNA binding domain by itself is not expected to induce any long-term effects, we next set out to test different epigenetic enzymes (DNMT3A and G9A). Fusion of epigenetic effector domains with ZFs resulted in the same magnitude of silencing as the ZF-SKD fusions, indicating that our approach worked as we aimed for. Furthermore, targeted DNA methylation or histone methylation has the advantage that its effect has the potential to be permanent (22, 24), albeit the stability and heritability of epigenetic editing is still controversial (10, 13). The particular local chromatin context of the targeted loci could influence the potency and longevity of the repression.

One limitation of our study is that most experiments were conducted in the alveolar cell line A549. Since we already showed convincing *MUC5AC* and *AGR2* silencing in A549 cells, it will be interesting to investigate whether this effect is also observed within the more relevant models of mucus hypersecretion in the future, such as using the air-liquid interface culture of the primary airway epithelial cells from patients with COPD. In addition, before use in the clinical setting, it is necessary to evaluate the off-target effects, such as the ZFs binding specificity and target cell specificity.

In summary, we successfully reduced mucus-related gene expression by targeted silencing of *SPDEF.* This new approach (epigenetic editing) has the potential to induce a permanent anti-mucus effect, which has implications for development of novel therapeutic strategies to treat patients with chronic mucus hypersecretion in the future.

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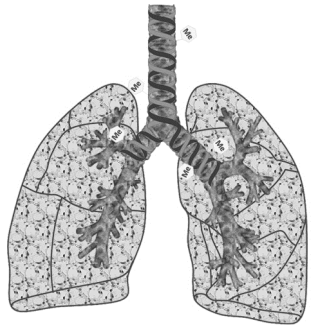
Chapter 6

Summary, general discussion and future perspective



Summary

Maternal smoking during pregnancy reduces lung function at birth and increases the risk for COPD in adulthood, and even synergizes with personal smoking to increase airflow limitation and COPD prevalence in adults. Epigenetic mechanisms contribute to COPD pathogenesis, and more knowledge regarding epigenetic regulation of a major phenotype within COPD (i.e. goblet cell differentiation) would provide novel targets for the development of epigenetic–based therapeutic strategies.

The overall aim of this thesis was to 1) assess the effects and the molecular mechanisms of maternal smoking during pregnancy on (smoke-induced) lung pathology in an experimental mouse model, 2) examine epigenetic mechanisms related to goblet cell differentiation and COPD, and 3) establish epigenetic editing as a tool to reduce mucus production.

Chapter 2 describes an experimental mouse study in which the effect of prenatal smoke exposure on airway epithelial cell development and underlying molecular mechanisms in *neonatal* offspring was investigated. Maternal smoking during pregnancy disturbed the mucociliary epithelium development in the neonatal offspring as it inhibited the ciliated cell numbers and expression of the major cilia-related transcription factor *Foxj1*. Our finding thus helps explaining the higher prevalence of fetal and childhood respiratory infections in case of maternal smoking. In addition, prenatal smoke exposed neonates had an increased expression of transcription factors *Spdef* and *Foxm1* which are involved in secretory cell differentiation, even though no goblet cells were present yet in the neonatal lung, explaining enhanced susceptibility for goblet cell metaplasia later in life. Moreover, our data on increased expression of the Notch target gene *Hey1* suggest that Notch signaling, balancer of the ciliated and secretory cell fates in developing airways may be disturbed by the prenatal smoke exposure.

In **Chapter 3** we continued our studies in this mouse model and asked whether *adult* offspring from mothers exposed to cigarette smoke during

pregnancy were more susceptible to (smoke-induced) inflammation and tissue remodeling in the lung. Changes in lung pathology were linked to expression of genes that are important in inflammation, xenobiotic metabolism, the antioxidant defense system, aging and senescence and genes that are related to tissue repair. This question was addressed in a mouse model in which maternal smoking during pregnancy (prenatal smoke exposure) was combined with postnatal smoke exposure in adult offspring. Interestingly, prenatal smoke exposure was found to down regulate smooth muscle thickening and affect gene expression related to inflammation, oxidative stress and repair. Expression of the anti-inflammatory gene *Ahr*, the anti-oxidant gene *Foxo3* and the anti-aging gene *Sirt1* were all down regulated in prenatal smoke exposed offspring, whereas the airway basal cell-related genes *Krt5* and *Trp63* were increased. Postnatal offspring smoking promoted tissue remodeling (more smooth muscle thickening and higher expression of *Muc5ac*), inflammation (M2 dominant macrophages) and higher gene expression of *Ahrr*, *Cyp1a1* and *Krt5*. Prenatal smoke exposure did not further deteriorate smoke-induced changes except for *Krt5* expression, for which less up regulation was found in offspring that was born from a smoke-exposed mother. This could implicate that endogenous repair mechanisms after injury are less effective when prenatally smoke exposed.

Chapter 4 describes the expression and methylation dynamics of *SPDEF* and *FOXA2* in an *in vitro* model (ALI culture) of airway epithelial cell differentiation using PBECs from individuals with COPD and controls. In control subjects, expression of *SPDEF*, together with *MUC5AC* and *AGR2*, was increased whereas *FOXA2* expression was decreased as expected during IL-13-induced goblet cell differentiation. This was associated with *hyper*methylation of CpG #8 in the SPDEF promoter whereas no methylation changes in the *FOXA2* promoter were found. In patients with COPD, expression of *SPDEF*, *AGR2*, and *MUC5AC*, as well as methylation of *SPDEF* demonstrated the same trends during IL-13-induced goblet cell differentiation as in controls, except for the expression and DNA methylation of *FOXA2*. In addition, the difference between COPD and

control was addressed in ALI- derived culture without the presence of IL-13. Interestingly, higher expression of *MUC5AC*, *AGR2* and *SPDEF* were found in COPD-derived ALI cultures when compared to controls, which was associated with *hypo*methylation of the *SPDEF* promoter. *FOXA2* expression was not different between COPD-derived ALI cultures and controls, even though COPD-derived ALI cultures displayed hypomethylation in the *FOXA2* promoter. These findings indicate that in COPD, goblet cell differentiation and mucus-related gene expression is aberrant and related to a change in DNA methylation levels of *SPDEF* and *FOXA2*. This aberrant DNA methylation might underlie the mucus hypersecretion in COPD, providing novel targets in the development of epigenetic-based anti-mucus therapeutic strategies.

Chapter 5 describes a promising epigenetic-based approach for inhibiting the mucus production using SPDEF as target. In this *in vitro* study, four zinc finger proteins were engineered to target the *SPDEF* promoter, and then fused to different effector domains (SKD, DNMT3A or G9a). The fusion proteins were transduced to A549 cells which highly express MUC5AC and SPDEF. All fusion proteins were found to be able to effectively suppress both SPDEF mRNA and protein expression, and ZFs-DNMT3A induced de novo DNA methylation at the *SPDEF* promoter. Importantly, all approaches were accompanied by inhibition of downstream mucus-related genes *AGR2* and *MUC5AC* expression. These findings open avenues for epigenetic editing as a novel therapeutic strategy to induce, potentially long-lasting, mucus inhibition.

General discussion

Smoke exposure effect on airway epithelial cell differentiation

Smoke exposure effect on ciliated cells

The ciliated cells of the airway epithelium play a critical role in proper mucociliary clearance of inhaled particles and pathogens. In previous studies, decrease of ciliated cell numbers, shortened airway cilia and a decreased ciliary beat frequency have been linked with impaired mucociliary clearance in smokers [1-3]. In chapter 2, we found decreased numbers of the ciliated cell and decreased expression of the major cilia-related transcription factor *Foxj1* in prenatal smoke exposed neonates, which might explain the increased infant and childhood hospitalization for respiratory infections [4,5]. In a recent mouse study of COPD, 9-12 months smoke exposure resulted in a total loss of cilia and ciliated cells in the trachea [3]. It is not certain however, whether our finding related to decreased presence of ciliated cells has the same underlying mechanism as the findings of loss of cilia and ciliated cells after chronic smoke exposure. In the COPD mouse study, reduction of ciliated cells seemed to be accompanied by an additional total loss of secretory cells, leaving only basal epithelial cells lining the interior of the trachea. In our model however, double staining of cells for CC10 and tubulin showed only a few epithelial cells negative for both markers (see also Figure 3 from Chapter 2). In addition, mRNA expression of basal cell markers *Trp63* or *Krt5* showed no difference between both groups but presence of KRT5 positive cells still needs to be confirmed by IHC.

Our data do support two recent *in vitro* studies in which PBECs were exposed to cigarette smoke extract (CSE) during differentiation at ALI [6,7]. Both of these studies showed CSE-mediated reduction of ciliated cell numbers and induction of basal cells (KRT14), even though these two studies show contrasting results regarding to secretory cells (as will be further discussed later). This suggests that cigarette smoke can alter the cellular composition of the airway epithelium by affecting the (basal) cell

differentiation. Besides that, Brekman *et al.* showed a broad CSE-mediated suppression of genes involved in ciliary biology, including transcription factors FOXJ1, regulatory factor X2 (RFX2), RFX3 and their targets genes. In our study, lower numbers of ciliated cells were accompanied by lower expression of *Foxj1*. Ciliated cell differentiation and ciliogenesis is a complex process in which a large network of genes, necessary for cilia assembly or function is involved. FOXJ1 and RFX family of transcription factors have been shown to be important players in controlling ciliary gene expression [8]. FOXJ1 regulates genes involved in cilia motility and the apical transport of basal bodies, whereas RFX proteins regulate genes involved in intraflagellar transport and in basal body anchoring. Both transcription factors share some common target genes involved in cilia motility. Overexpression of FOXJ1 was shown to be able to prevent CSE-mediated cilia shortening but did not prevent CSE-mediated decrease in ciliated cell number [6], indicating a potential therapeutic target, only in the early stage.

It is of note that the cytological and functional alteration caused by the cigarette smoking might be not permanent which could be covered by the continuous regeneration of the respiratory epithelium when the irritating stimulus of cigarette smoking is removed [9]. In our mouse model described in Chapter 3, prenatal smoke exposure in combination with 12 weeks postnatal smoke exposure had no effect on either expression of *Foxj1* or ciliated cell numbers (data not shown). This might suggest that the airway epithelium has recovered from the earlier observed prenatal smoke exposure-induced inhibition in ciliated cell development during postnatal lung development, and that 12 weeks of postnatal smoke exposure might not be enough to severely damage the epithelium as shown after 6-12 months of smoke exposure [3]. In chapter 4, we found no difference for *FOXJ1* expression in COPD- and control- derived ALI- cultures in a small set of experiments.

Notch signaling has been reported to promote secretory cell development over ciliary cell fate [10,11]. In chapter 2, we show that prenatal exposure

to cigarette smoke up regulates *Hey1* expression. *Hey1* is a Notch target gene which implies active Notch signaling in offspring from smoke-exposed mothers. Down regulation of Notch downstream effector genes was shown in small airway epithelium of smokers and even more down regulation in smokers with COPD support our finding [12]. Therefore, active Notch signaling could have contributed to the lower ciliated cell development and increased expression of goblet cell gene markers.

Smoke exposure effect on Club (Clara) cells and goblet cells

In chapter 2, no changes were found with respect to the number of Club cells and gene expression of *Scgb1a1* (*Cc10*) in prenatal smoke-exposed neonatal mice, whereas increased expression of transcription factors involved in secretory cell differentiation *Spdef* and *Foxm1* were found, even though goblet cells were not present yet in the neonatal lung. In chapter 3, we showed increased expression of *Muc5ac* in postnatal smoke exposed mice, which is in agreement of a higher risk for chronic mucus hypersecretion in smokers [13]. However, no PAS positive goblet cells were observed and gene expression of *Scgb1a1* was not changed (data not shown). Our data are in agreement with an interesting study from Adair-Kirk et al [14] who found no difference in the number of Club cells and ciliated cells after 6 months of CS exposure but did find an upregulation of the *Cyp1b1* gene in Club cells due to smoking. This assigns the Club cell to be important in the detoxification of CS. In contrast to our results, a reduction in Club cell numbers has been found in smoke-exposed rats [15], prenatal smoke-exposed mice (secondhand smoke) [16], and also *in vitro* CSE-mediated ALI cultures [6], whereas another *in vitro* CSE-mediated ALI cultures showed increased numbers of Club cells and goblets cells [7]. The discrepancies between our study and others regarding smoke exposure induced Club cell number changes might reflect species-specific differences, *in vitro* and *in vivo* difference, cigarette and smoke system difference.

Future perspectives

The studies presented in this thesis leave us with some questions but also suggest several possibilities for future research.

Sex and gender differences in COPD

There has been a rapid increase in the numbers of female patients with COPD over the past 20 years [17]. Despite the fact of the increasing numbers of female smokers, the gender difference in the susceptibility to the effect of cigarette smoke is still a topic for debate. Multiple studies have shown that female smokers have a faster annual decline in lung function (FEV1% predicted) than male smokers [18,19], whereas females also show a quicker recovery from lung function decline when they quitted smoking [19]. In addition, females were shown to have a lower lung function and more severe disease in the group of patients with COPD with low smoking exposure or early-onset of disease [20]. Also, in the majority of patients (around 70%) with severe COPD, early-onset COPD was seen in females [21,22] even though females had markedly fewer pack-years of smoke exposure [23]. The differential susceptibility to cigarette smoke in females and males could be explained by the hormonally mediated differences in cigarette-smoke metabolism or by the fact that females have smaller lungs and airways than males [24]. Recently a mouse model of COPD showed that chronic smoke exposure increased small airway remodeling in female compared with male mice, and that this effect was prevented by ovariectomy or selective estrogen antagonism with tamoxifen, providing evidence that estrogen may be involved in the dysregulation of the oxidant/TGFβ-signaling axis that is unique in smoke-exposed female mice [25].

In our (maternal) smoke mouse model (chapter 3), we found sex differences for multiple parameters in the response to smoke exposure, which varied from a response in only one sex, an opposite effect in both sexes or a stronger effect in one sex than in the other. For *Muc5ac* expression, a postnatal smoke effects was only found in males (Figure 1a),

whereas the number of neutrophils only increased in females in pre- and postnatal smoke exposed offspring (Figure 1b). In addition, *Ahr* gene expression in males was down regulated due to smoke exposure, whereas *Ahr* gene expression was up regulated in females (Figure 1c). Finally, the smoke effect on *Cyp1a1* gene expression was much larger in females then in males (Figure 1d).

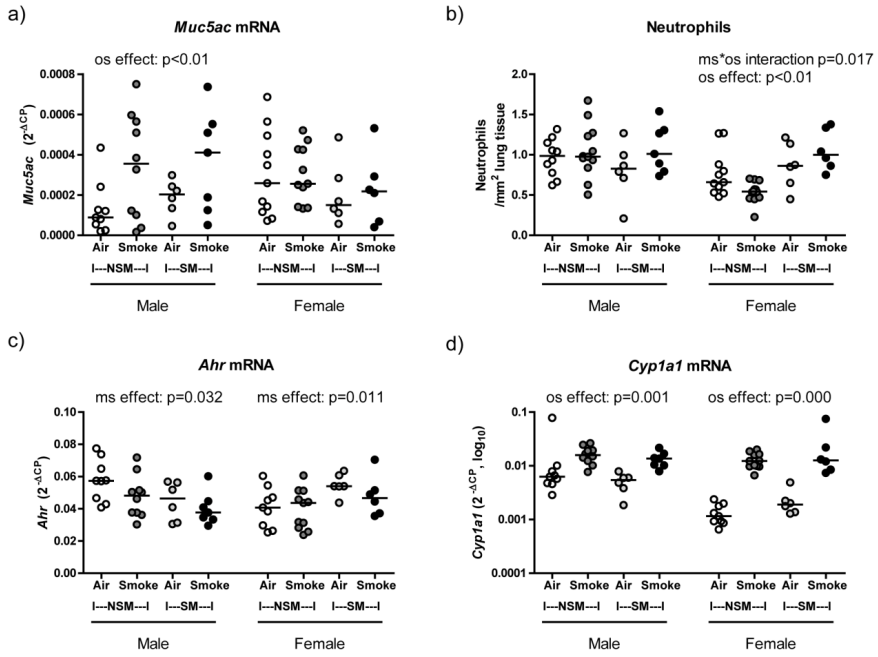


Figure 1 Different (prenatally) smoke-exposure effects on expression of* Muc5ac, Ahr, Cyp1a1 *and number of neutrophils in male and female mice. *Expression of (a)* Muc5ac*, (c)* Ahrr*, and (d)* Cyp1a1 *was analyzed by quantitative Real Time-PCR in RNA, isolated from lung tissue. (b) Scores of Neutrophils (GR1) in lung tissue. Data represent medians of expression or cell numbers. 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. (A) Negative interaction maternal smoke and offspring smoke p=0.017 from linear regression analyses indicates that the offspring smoke exposure effect on decreased neutrophil numbers was weaker in offspring from smoking mothers.*

As can be found in the Estrogen Responsive Genes Database (ERGDB) and Androgen Responsive Gene Database (ARGDB), putative estrogen response elements are shown in the promoter sequence of different genes, including *Cyp1a1, Cyp1b1, Trp63, Foxj1,* and *Scgb1a1*, whereas there are also some potential androgen responsive genes in our study such as *Krt5*, *Ahr*, and *Spdef*. Altogether it suggests the involvement of hormonally mediated differences in multiple biology processes. Recently, smoke was shown to be able to affect the expression and activity of estrogen receptor- alpha (ERα) [26]. In addition, recent studies have shown that activated AHR induced the recruitment of ERα to AHR-regulated genes and that AHR is recruited to ERa-regulated genes [27,28], which might also explain the sex difference in AHR-related xenobiotics metabolism and lung inflammation [29,30]. Further exploration of sex differences and the underlying mechanisms would be of high interest and importance.

Cross-talk between transcription factors and DNA methylation states – in airway epithelial cell differentiation and COPD

In chapter 4, we observed variable association between *SPDEF* mRNA expression and DNA methylation of different CpG sites in the *SPDEF* promoter during *in vitro* airway epithelial cell (AEC) differentiation. First, methylation of CpG #8 was increased during the goblet cell differentiation, which was positively correlated with *SPDEF* mRNA expression changes. The same trend of *SPDEF* expression and methylation changes were found during IL-13-promoted goblet cell differentiation using cells from five patients with COPD and six control subjects. These changes might due to goblet cell differentiation but cannot rule out the direct effect of IL-13 treatment itself, which is supported by a recent study in IL-13 treated AECs from asthma patients [31]. This study showed that an altered global DNA methylation pattern was induced by 24 hours of IL-13 treatment. Interestingly, this IL-13 epigenetic signature was validated in freshly isolated AECs from subjects with asthma and associated with asthma severity and lung function or eosinophilia, which underlines the importance

of IL-13 in allergic asthma [31]. However, from that study it is not known whether our genes of interest were affected. We speculate that NKX2-1 is a transcriptional inhibitor of *SPDEF* and the NKX2-1 inhibition of the *SPDEF* promoter activity might be impaired by DNA methylation of CpG #8 where putative NKX2-1 binding sites are located. For now, there is no direct evidence showing that *SPDEF* is a target gene of NKX2-1 except for their inverse role in goblet cell differentiation and mucus production. It would be interesting to further investigate the relation of NKX2-1 binding and *SPDEF* promoter methylation (particularly for CpG #8) to *SPDEF* promoter activity, using Chromatin Immunoprecipitation assays and the *SPDEF* promoter-driven luciferace assay, combined with Site-directed Mutagenesis, *in vitro* DNA Methylation and *in vitro* NKX2-1 overexpression in the future. Second, hypomethylation of CpG #6 was shown in the COPD-derived ALI-cultures (14 days) compared to the control-derived ALI-cultures, which was negative associated with SPDEF mRNA expression changes. This observation is consistent with the classically repressive role of DNA methylation in the regulation of transcription. However, as the results came from a small number of patients with COPD versus controls, this finding warrants replication in another set of patients with COPD. It is also interesting to examine whether the differential *SPDEF* expression and methylation of CpG #6 is smoke-related or COPD-disease specific. To answer this question, cells from healthy nonsmokers, healthy smokers and COPD smokers should be tested in the future. Another important question to be addressed is whether SPDEF expression and methylation are related to mucus hypersecretion or lung function (as a biomarker). For this, (airway epithelium brushings/ biopsies or sputum) from COPD patients with or without chronic mucus secretion should be compared.

In the study described in chapter 4, we also observed differential *FOXA2* methylation in COPD-derived ALI-cultures compared to control-derived ALI-cultures, whereas transcriptional expression was not changed. Again, for this, we need validation of our data in another replication set of patients. Because the changes of *FOXA2* methylation were quite small (max 4%), the next question to address is whether this small difference was driven by only

a small increase of goblet cell numbers. Using purely isolated population of goblet cells, Club cells and ciliated cells might help to dissect this question. It is also necessary to investigate whether the methylation changes are smoke-dependent, because *FOXA2* expression was shown to be decreased in the airway epithelium of healthy smokers and smokers with COPD compared to nonsmokers [32]. The correlation between *FOXA2* and *SPDEF* is also interesting and there is no direct evidence yet, although evidence exists on mutually inhibition of each other [33]. Furthermore, putative binding sites of FOXA2/1 locate in the *SPDEF* promoter and putative binding sites of SPDEF exist in the promoter and first exon of *FOXA2*. All of this allows us to speculate that SPDEF and FOXA2 can directly regulate each other, and could form a feedback loop in goblet cell differentiation and mucus production.

Gene targeted epigenetic reprogramming in the treatment of asthma and COPD

In chapter 2, we found that prenatal smoke exposure induced *Spdef* expression in the lung of neonates. Furthermore, in chapter 4, we found that increased *SPDEF* expression was associated with increased *MUC5AC* expression during goblet cell differentiation as well as in COPD-derived ALI-culture when compared to controls. This was accompanied with differential methylation changes in the *SPDEF* promoter. As SPDEF is a known key transcription factor required for airway goblet cell differentiation and mucus production in mice and in human primary airway epithelial cells in vitro [33], SPDEF is a potential therapeutic target for anti-mucus production. In Chapter 5 we applied the novel approach of epigenetic editing, in which programmable Zinc Finger (ZF) DNA binding proteins were constructed to target various effectors (DNA methyltransferase 3A (DNMT3A), histone 3 lysine 9 (H3K9) methyltransferase G9a and the transcription repressor Super KRAB Domain (SKD)) to the promoter sequences of *SPDEF*. Using this new approach we were able to downregulate MUC5AC mRNA and protein expression in human alveolar

epithelial cell line A549 cells. In addition, we induced DNA methylation on the *SPDEF* promoter, which bears the potential to maintain the SPDEF silencing and mucus inhibition. So, as next steps we are going to investigate the sustainability effect of ZF-DNMT3A on the SPDEF silencing and mucus inhibition using transient expression of ZF-DNMT3A, compared with catalytic mutant of ZF-DNMT3A, and ZF-SKD. Previous studies have shown that long-term stable repression of the tumor suppressor gene *MASPIN* and oncogene *SOX2* could be achieved by ZF targeted DNMT3A in breast cancer cells even after the ZF-DNMT3A was not expressed anymore, which proves that targeted DNA methylation is a hit-and run strategy for long-lasting epigenetic reprogramming gene expression and cell state [34,35]. However, another recent study showed that DNA methylation or H3K9 methylation targeted into the VEGF-A promoter was not stably propagated after the loss of expression of the ZF targeted DNA and H3K9 methyltransferases [36]. One possible explanation of the discrepancy between these studies is the chromatin context of the tested loci. This means that the choice of epigenetic editor for long-term silencing is dependent on the target gene and the target cell type. With respect to this issue, the next question is to address whether the SPDEF silencing and the MUC5AC inhibition effect driven by the ZF-effectors is reproducible in other model systems, because A549 is a human alveolar epithelial cell line which might present a chromatin context different from our ultimate target: bronchial epithelial cells from human individuals with mucus hypersecretion. To best mimic our target cells, we continued our experiments in primary bronchial epithelial cells (PBECs). For this pilot study (Figure 2a), PBECs from one donor were seeded into the transwell inserts in hormone/growth factor–deprived medium (Bronchial Epithelial Cell Growth Medium, BEGM) together with lentiviral delivered ZF-SKD treatment for two days. Then medium was replaced with BEGM containing puromycin to enrich the positively transduced cells for five days. Next, cells were cultured in normal BEGM until cells reached confluence to create ALI and subsequently they were allowed to differentiate in normal BEGM with IL-13 (1 ng/ml) for 21 days. Some promising preliminary results showed down regulation of SPDEF

transcription and lower numbers of MUC5AC positive cells (Figure 2b-d). However, our next attempt using another three donors treated with ZF-DNMT3A and ZF-G9a was not successful because of too few cells that were left after puromycin treatment, making it impossible for the cells to reach confluence. Two possible explanations are (1) the existence of a donor-specific sensitivity for puromycin, and (2) the size of ZF-DNMT3A and ZF-G9a constructs might be too large for efficient lentiviral transduction, leading to less positively transduced cells left in the latter experiment. Improvement of the viral titers would increase the transduction efficiency.

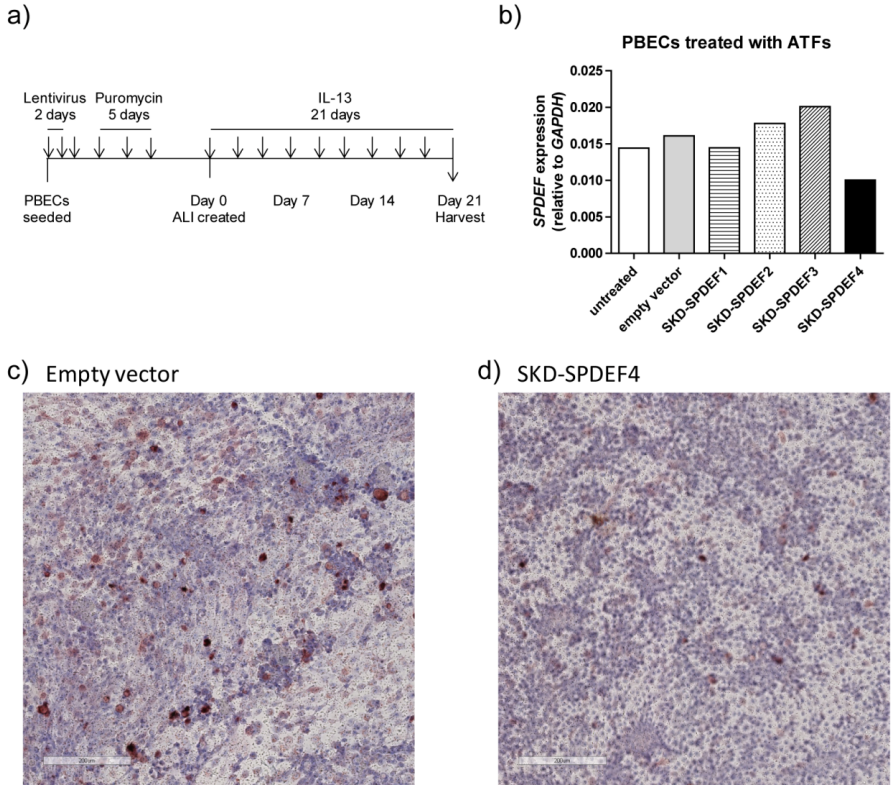


Figure 2* SPDEF-*targeted silencing by artificial transcription factors (ATFs) in primary bronchial epithelial cells (PBECs). *a) Schematic representations of the experimental model. PBECs from one donor were seeded into the transwell inserts in BEGM medium together with lentiviral delivered ZF-SKD treatment for two days and then cells were refreshed with BEGM medium. Four days after the cell were seeded, cells were cultured with BEGM with puromycin to enrich the positively*

transduced cells for five days. Next, cells were cultured in normal BEGM until cells reached confluence to create ALI and subsequently they were allowed to differentiate in normal BEGM with 1 ng/ml IL-13 for 21 days. Cells were harvested for RNA, and morphology analysis. mRNA expression of SPDEF *(b) was analysed by real-time quantitative PCR. Representative images of immunocytochemistry staining on MUC5AC (red) at ALI day 21 for empty vector (c) and SKD-SPDEF4 (d) treated cells.*

Regarding the delivery approach for future experiments, adeno-associated virus (AAV) or adenovirus based delivery would be a better choice than lentiviral based delivery used in this thesis. First, AAVs or adenoviruses do not require hazardous viral DNA/RNA integration into the host DNA [37] which makes it safer for gene therapy and clinical use, as supported by the recent approval of the first, and the only one till now, gene therapy drug for lipoprotein lipase deficiency treatment (AAV based) [38]. Second, for the basic biological studies, these viruses cannot propagate in the host cells and are degraded after some time [39], which makes it feasible to follow the dynamics of the establishment and loss of the epigenetic marks and to investigate the sustainability of the epigenetic editing approach. Next to the viral delivery, the direct delivery of the targeted Epigenetic-Effectors as proteins [40] or chemically modified mRNA [41] also proved to be quite promising. Further step into the in vivo pulmonary delivery, mucolytic nanoparticles will be proper vehicle for the delivery of gene constructs into bronchial epithelial cells after nanoparticle inhalation by aerosolization.

With respect to the gene specificity of the targeting platform, engineered zinc finger binding was shown, by us and by others, to be wide spread for several ZF-constructs [42]. The novel target platform transcription activator-like effectors (TALEs) and the clustered regularly interspaced short palindromic repeats (CRISPRs)-dCas9 complex (where the nuclease activity of Cas is mutated to result in a dead Cas9 (dCas)) were shown to have a higher specificity [43-45]. The CRISPR-dCas9 system is particularly suitable to high-throughput screens because its targeting is based on a cheap and flexible system where a single guide RNA pairs with one strand of its target DNA. However, ZFs have the advantage that these proteins are of human

origin and may demonstrate less immunogenicity in clinical application than TALE and CRISPR/ Cas9 system (of bacterial origin). Indeed, several ZF-constructs are currently tested in clinical trials for genome editing applications.

In conclusion, in this thesis we have shown that prenatal smoke exposure inhibited ciliated cell development and increases expression of secretory cell differentiation related transcription factors *Spdef* and *Foxm1* in newborns' lung. In adult mice however, prenatal smoke exposure affected mostly other gene expression pathways such as pathways related to oxidative stress, aging xenobiotic metabolism, aging and lung repair. Postnatal smoke effects on inflammation, remodeling and xenobiotic metabolism seemed hardly affected by prenatal smoke exposure effects. Regarding our in vitro experiments, in cultured primary cells of patients with COPD, DNA hypomethylation and increased expression of *SPDEF* were associated with increased expression of *MUC5AC* in airway epithelial cells. Targeted silencing of SPDEF using Zinc Finger proteins directed DNA and histone methyltransferase was able to induce the inhibition of both MUC5AC mRNA and protein. This opens avenues for modulating expression of any desired target gene. Further research however, needs to explore the specificity and sustainability of the targeted epigenetic marks, and the ultimate goal would be to achieve sustained reprogramming (e.g. mucus reduction) *in vivo*.

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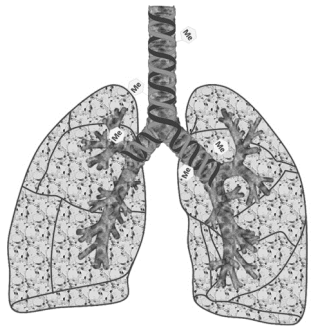
Appendices



Nederlandse samenvatting

Inleiding

Ondanks vele aanwijzingen dat roken tijdens de zwangerschap schadelijk is voor de groei en ontwikkeling van het ongeboren kind, de foetus, blijft 30% van de vrouwen roken tijdens de zwangerschap. Bevolkingsonderzoek laat zien dat blootstelling aan rook tijdens de zwangerschap het risico verhoogt op ontwikkeling van astma op de kinderleeftijd. Daarnaast lijkt roken tijdens de zwangerschap een risicofactor te zijn voor ontwikkeling van de chronische rook-gerelateerde ziekte COPD later in het leven. Dit suggereert dat de kiem voor het ontstaan van COPD op volwassen leeftijd al gelegd wordt tijdens de ontwikkeling in de baarmoeder. Risicofactoren op een bepaalde ziekte kunnen erfelijk zijn en liggen dan vast in ons erfelijk materiaal, ons DNA. Daarnaast kunnen tijdens het leven veranderingen optreden in het gebruik van het DNA, zogenaamde epigenetische veranderingen. Epigenetische mechanismen zoals veranderingen in DNA methylatie en modificaties van het DNA-bindende eiwit histon zouden daaraan ten grondslag kunnen liggen. Meer kennis over de epigenetische mechanismen bij COPD zou nieuwe targets kunnen opleveren voor de ontwikkeling van epigenetisch gebaseerde medicijnen.

Doel van het onderzoek

Het algemene doel van het onderzoek beschreven in dit proefschrift was 1) het in kaart brengen van de moleculaire effecten van roken van de moeder tijdens de zwangerschap op de (rook-geïnduceerde) longschade in het nageslacht in een experimenteel muismodel, 2) onderzoek doen naar epigenetische mechanismen die verband houden met de verhoogde ontwikkeling van slijm producerende cellen en slijmproductie in de long bij personen met COPD, en 3) het testen van herschrijven van epigenetische veranderingen (‘epigenetische editing’) als een instrument om de slijmproductie te verminderen.

Epigenetica

Epigenetica verwijst naar de moleculaire mechanismen die ten grondslag liggen aan een "stabiel overerfbaar fenotype als gevolg van veranderingen in een chromosoom, zonder veranderingen in de DNA-sequentie (volgorde van de baseparen, ook wel genotype genoemd)". De term "epigenetics" werd oorspronkelijk bedacht door C. H. Waddington. Hiermee verklaarde hij de observatie dat een stamcel met behoud van zijn genotype, kan uitgroeien (differentiëren) tot verschillende celtypen met een verschillende functie (fenotype). Als zodanig is de epigenetica belangrijk voor het handhaven van genexpressie patronen (fenotype) tijdens celdelingen gedurende de levensduur van de cel, en zelfs gedurende meerdere generaties van organismen. Epigenetische processen spelen dus een belangrijke rol in genregulatie, en recente studies hebben de relevantie van epigenetische disregulatie aangetoond als onderliggend voor diverse ziekten waaronder astma en COPD.

In de kern van een cel wordt het chromatine compact gehouden doordat DNA strak gewikkeld is rond histoneiwitten. Wijzigingen in aanwezigheid van epigenetische 'markeringen' zoals methylgroepen rond het DNA, kunnen de expressie van genen beïnvloeden. Wanneer een gen op bepaalde posities veel methyl groepen heeft zal dit gen niet functioneel zijn (er wordt geen eiwit gevormd).

Roken tijdens de zwangerschap

Bevolkingsonderzoek laat zien dat nakomelingen van moeders (maar ook vaders) die rookten tijdens de zwangerschap een grotere kans hebben op het krijgen van infecties in de long, benauwdheid en astma. Onderliggend hieraan is dat blootstelling aan sigarettenrook tijdens de zwangerschap de longontwikkeling van de ongeboren vrucht verstoort waardoor deze kinderen met een slechtere longfunctie geboren worden. Een slechte longfunctie is een risico factor voor het krijgen van astma en COPD later in het leven. Daarnaast is in proefdiermodellen aangetoond dat blootstelling

aan sigarettenrook tijdens de zwangerschap effect heeft op de expressie van genen die belangrijk zijn voor een juiste ontwikkeling van de long. Dit zijn grotendeels dezelfde genen die belangrijk zijn bij longweefselherstel later in het leven. Vroege blootstelling vóór de geboorte heeft dus hiermee potentiële gevolgen op lange termijn die van invloed kunnen zijn voor de ontstaansmechanismen van een longziekte op de volwassen leeftijd. Het risico op COPD wordt nog verder verhoogd als vroeg blootgestelde personen zelf ook gaan roken of blootgesteld worden aan omgevingsrook of andere luchtvervuiling.

Hoofdstuk 2 beschrijft een experimentele studie in muizen waarbij het effect van blootstelling aan sigarettenrook tijdens de zwangerschap op de ontwikkeling van het luchtwegepitheel, de laag cellen die de bekleding vormt van de luchtwegen, wordt onderzocht in nakomelingen. Deze studie liet een verstoorde epitheelcel ontwikkeling zien in pasgeboren nakomelingen van moeders die blootgesteld waren aan rook tijdens de zwangerschap. Deze pups hadden minder trilhaardragende cellen in de luchtwegen en het lagere aantal cellen ging gepaard met een lagere genexpressie van het gen *Foxj1* dat belangrijk is voor de aanmaak van trilharen. Trilhaardragende cellen helpen de long schoon te houden aangezien ze met hun trilharen slijm, stof, microben en andere schadelijke stoffen naar de mond helpen te transporteren. Wanneer slijm aanwezig blijft vormt het een risico voor het ontstaan van infecties. Onze bevinding ondersteunt hiermee de observatie van het vaker voorkomen van luchtweg infecties bij jonge kinderen van een rokende moeders. Bovendien hadden rook-blootgestelde pups een hogere expressie van de genen *Spdef* en *Foxm1*, genen die betrokken zijn bij de aanleg en ontwikkeling van de slijm producerende cel, ook wel slijmbekercel genoemd. Hoewel er nog geen slijmbekercellen aanwezig waren in de longen van de pasgeboren muizen kan deze bevinding wel de verhoogde gevoeligheid voor het ontstaan van deze slijmbekercellen verklaren op latere leeftijd.

In **hoofdstuk 3** werden de studies in dit muismodel voortgezet met aanvullende rook blootstellingen aan volwassen nakomelingen van

moeders die blootgesteld waren aan sigarettenrook tijdens de zwangerschap. Hierbij was de vraag of vroeg blootgestelde nakomelingen extra gevoelig waren voor ontsteking en weefselverlittekening, ook wel remodelering genoemd, in de longen nadat ze zelf ook gedurende 3 maanden aan rook waren blootgesteld op volwassen leeftijd. Veranderingen in de long werden gekoppeld aan expressie van genen die belangrijk zijn bij ontsteking, het antioxidant systeem, longweefselherstel, veroudering en genen die gerelateerd zijn aan het onschadelijk maken van schadelijke stoffen (sigarettenrook) in het lichaam. Uit deze studie bleek dat volwassen nakomelingen van rokende moeders, ongeacht of ze zelf ook aan rook waren blootgesteld of niet, een lagere expressie hadden van genen die beschermend zijn met betrekking tot het ontwikkelen van ontsteking, oxidatieve stress en veroudering, en een hogere expressie van genen die belangrijk zijn voor longweefselherstel.

Rookblootstelling van de nakomelingen zelf bevorderde weefselremodelering (meer verdikking van gladde spieren en hogere expressie van het slijm-gerelateerde gen *Muc5ac*), ontsteking (verhoogd aantal macrofagen, dit is een bepaald type witte bloedcellen belangrijk voor opruimen van rookdeeltjes) en hogere genexpressie van genen die belangrijk zijn bij weefselherstel en het onschadelijk maken van schadelijke stoffen. Vroege blootstelling aan rook tijdens de zwangerschap verergerde deze effecten in de meeste gevallen niet.

Chronische slijmoverproductie en COPD

COPD is een ongeneeslijke, progressieve longziekte die zich manifesteert in de grote (chronische bronchitis) en kleine luchtwegen en de longblaasjes (emfyseem). Blootstelling aan sigarettenrook is de belangrijkste risicofactor voor het ontwikkelen van COPD. Chronische bronchitis gaat gepaard met chronische aanwezigheid van ontstekingscellen en overproductie van slijm in de luchtwegen. Bij rokers en veel personen met COPD is deze overproductie van slijm een belangrijke oorzaak van klachten zoals hoesten

en benauwdheid. Het slijm wordt geproduceerd door slijmbekercellen in de grote luchtwegen. Slijmbekercellen ontstaan vanuit de normale luchtwegepitheelcellen na langdurige blootstelling aan sigarettenrook.

In de epitheelcel zijn verschillende signaleringsroutes beschreven die de verandering naar een slijmbekercel en slijmproductie bevorderen. Gronings onderzoek in rokers zonder een longaandoening en personen met COPD heeft aangetoond dat een aantal verschillende genen verhoogd tot expressie komen in epitheel van rokers en personen met COPD die hoesten en te veel slijm produceren. Een van de gevonden genen, het gen *SPDEF* heeft hierbij een centrale rol en stuurt een heel netwerk van genen aan. *SPDEF* bevordert hiermee de *ontwikkeling* van slijmbekercellen, als ook slijm*productie*. De productie van eiwitten zoals SPDEF wordt gereguleerd door epigenetische mechanismen waarbij een gen 'aan' of 'uit' gezet kan worden.

In **hoofdstuk 4** waren we geïnteresseerd of I) slijmbekercel uitgroei (differentiatie) gepaard gaat met een verandering in DNA methylatie van *SPDEF* en II) of deze epigenetische regulatie van *SPDEF* anders is in luchtwegepitheelcellen van personen met COPD dan bij controle personen. Hiertoe zijn ongedifferentieerde luchtwegepitheelcellen van personen met COPD en controles gedurende vier weken gekweekt tot de cellen waren uitgegroeid tot slijmbekercellen en trilhaardragende epitheelcellen. Deze uitgroei werd gevolgd door wekelijks een monster te nemen dat werd onderzocht op genexpressie en DNA methylatie. In deze studie hebben we aangetoond dat epitheelcel differentiatie onder invloed van toevoeging van de signaalstof interleukine 13 (IL-13), ter bevordering van slijmbekercel differentiatie, gepaard gaat met een toename van expressie van genen die betrokken zijn bij slijm productie zoals *MUC5AC* en *SPDEF*. Het gen *FOXA2* dat *SPDEF* expressie remt kwam lager tot expressie. Interessant was dat slijmbekerceldifferentiatie en toename van *SPDEF* genexpressie was geassocieerd met een verandering van de DNA methylering binnen het *SPDEF* gen. Dit was, waarschijnlijk door de toevoeging van IL-13 aan het kweeksysteem, niet verschillend voor COPD in vergelijking met de controle

cellen. Interessant genoeg werden er wel verschillen gevonden wanneer het experiment herhaald werd zonder de toevoeging van IL-13 in het kweeksysteem. Cellen van personen met COPD hadden na 14 dagen gekweekt te zijn een hogere expressie van *SPDEF* en *MUC5AC* en dit ging gepaard met een lagere methylatie van *SPDEF*. Deze studie laat daarmee zien dat de epigenetische mechanismen met betrekking tot *SPDEF* verstoord zijn in COPD en daarmee belangrijk en verklarend kunnen zijn bij de ontwikkeling van slijmbekercellen en slijmproductie.

In **hoofdstuk 5** beschrijven we een mogelijke vorm van therapie gebaseerd op onze bevindingen in de eerdere hoofdstukken. Het doel was DNA methylering van het *SPDEF* gen te verhogen en zodoende slijmproductie te verminderen. Dit is gelukt in de humane longcellijn A549 die net als primaire cellen van controles en personen met COPD de genen *SPDEF* en *MUC5AC* tot expressie brengt en slijm produceert. In deze studie kon *SPDEF* transcriptie negatief gereguleerd kunnen worden in de endogene situatie met behulp van DNA bindende domeinen (zinkvingers), die gemaakt waren om aan het SPDEF gen te binden, en vervolgens gefuseerd waren aan transcriptieregulerende eiwitten. Deze bevindingen openen mogelijkheden voor het epigenetisch, en dus mogelijk blijvend, veranderen van het *SPDEF* gen als nieuwe therapeutische strategie voor het remmen van slijmproductie.

Algemene conclusies van dit proefschrift

De studies die beschreven staan in dit proefschrift laten zien dat vroege blootstelling aan sigarettenrook tijdens de zwangerschap trilhaarcelontwikkeling remt en expressie van het slijmbekercel gerelateerde gen *Spdef* verhoogt in de longen van pasgeboren muizen. Echter, in volwassen muizen had vroege blootstelling aan rook tijdens de zwangerschap effect op genen met een andere functie zoals betrokkenheid bij oxidatieve stress, veroudering en longweefsel herstel. Rookblootstelling

van de nakomelingen zelf had daarentegen effect op ontsteking, remodelering, veroudering en genen die betrokken zijn bij het onschadelijk maken van toxische stoffen. Dit effect werd niet verder verstrekt door vroege blootstelling tijdens de zwangerschap.

Met behulp van celkweken van longcellen van personen met COPD werd vastgesteld dat het gen *SPDEF* een belangrijke rol speelt bij het ziektemechanisme en dus een goede target voor therapie zou kunnen zijn. *SPDEF* DNA methylering bleek verstoord te zijn en was hiermee een goede kandidaat voor epigenetische benadering met behulp van epigenetische editing. In een eerste studie in een cellijn bleek inderdaad dat gerichte verhoging van methylatie van het *SPDEF* gen leidde tot een remming van *SPDEF* transcriptie en slijmproductie. Dit veelbelovende resultaat is een eerste stap en opent perspectieven voor het moduleren van expressie van ieder gewenst gen voor de toekomst. Verder onderzoek moet echter eerst de specificiteit en de duurzaamheid van de epigenetische modulatie vaststellen, met het uiteindelijke doel om blijvende herprogrammering (bijvoorbeeld slijmreductie) in het organisme te bewerkstelligen.

中文摘要

前言

尽管有许多研究表明，怀孕期间吸烟有害于胎儿的生长发育，但仍有30％的女性在怀孕期间吸烟。目前已有流行病学研究表明，孕妇在怀孕期间吸烟会增加儿童患哮喘的风险。此外，产前的吸烟暴露可能会成为日后生活中胎儿患慢性堵塞性肺疾病（Chronic Obstructive Pulmonary Disease, COPD）的危险因素。这表明，在成年期发展为COPD 的危险可能起始于胎儿在子宫的发育过程。此外，已有研究表明：特定疾病的危险因素是可以遗传的，可以存在于我们的遗传物质——DNA 中。而且随着我们的生长发育，我们的 DNA 修饰也可以随之发生变化，即所谓的表观遗传变化。表观遗传学机制，比如 DNA 甲基化的变化和 DNA 结合蛋白（组蛋白）修饰的改变，可能用于解释患 COPD 这种疾病的危险因素的遗传性。综上所述，针对 COPD 的表观遗传学机制的研究可为对基于表观遗传学的 COPD 治疗的药物研发提供新的靶标。

研究目的

本论文的研究总体目标主要包括以下几方面：1）利用小鼠抽烟实验模型，研究母鼠孕期吸烟对子代肺损伤在分子学水平的影响；2）研究与COPD 患者肺组织中粘液细胞数量增加和粘液过度分泌相关的表观遗传学变化；3）通过改变病人的表观遗传学特征（“表观遗传学编辑”），以减少 COPD 患者肺组织中粘液的产生与分泌。

表观遗传学

表观遗传学是指在基因的核苷酸序列（碱基对的序列，也叫基因型）不发生改变的情况下，研究基因表达（表型）发生的可遗传性变化的分子学机制。“表观遗传学”这一概念最初是由 CH 沃丁顿提出。他利用这个机制来解释干细胞在保持其基因型的同时，可成长分化成具有

不同功能（表型）的不同类型细胞的现象。同理，表观遗传学机制对于在生物体一生中细胞分裂前后，甚至于几代生物体之间细胞保持其表型（基因表达）很关键。因此，表观遗传学在基因调控中起着极其重要的作用。近期的研究结果也表明，表观遗传学机制失调与多种疾病包括哮喘和 COPD 等的发生具有相关性。

在细胞核中，DNA 紧紧缠绕在组蛋白上，形成一种称为染色质的紧凑结构。表观遗传学“标签”（例如 DNA 甲基化和组蛋白修饰）的变化，可以影响基因的表达。基因在其某特定位置的甲基化改变可以阻止或促进其翻译表达过程。

怀孕期间吸烟

流行病学的调查结果显示，在怀孕期间吸烟的母亲（或父亲）的子代患肺部感染，呼吸急促和哮喘的机率更大。其潜在的机制是，在怀孕期间，香烟烟雾暴露会影响胎儿的肺发育，使得这些儿童在出生时具有较差的肺功能；而较差的肺功能是日后生活中发展为 COPD 和哮喘的危险因素。此外，动物实验模型的研究结果表明，在怀孕期间暴露于香烟烟雾，会影响那些与肺的正常发育密切相关的基因的表达。同时，大多数的这些基因在以后的生活中对肺组织损伤的修复也起着十分重要的作用。所以，怀孕期间（香烟）烟雾暴露具有潜在的长期后果，可能会影响子代成年后肺部疾病的发生。并且值得注意的是，对于早期（香烟烟雾）暴露的个体，在日后生活中存在的本身暴露于香烟或环境烟雾或其他的空气污染会进一步增加其罹患 COPD 的风险。

第 2 章着重阐述了在小鼠的抽烟模型中妊娠期间香烟烟雾暴露对于气道上皮的发育分化的影响。我们发现，在怀孕期间吸烟的母鼠的新生子代小鼠的气道上皮细胞的分化发育受到了影响，这些幼仔在呼吸道中具有较少的纤毛细胞。已有研究证实，Foxj1 基因对纤毛的生长有重要的调控作用；而且我们的研究结果也表明，纤毛细胞的数量减少和 Foxj1 基因表达水平的降低相关。此外，纤毛细胞有助于保护肺部，由于纤毛的规律性振动有助于运送肺中存在的粘液、灰尘、微生物等有害物质到口中。当粘液滞留在气道中时，会增加感染的发生风险。因

此，我们的研究结果也与吸烟母亲的年幼孩子具有较高的感染发病率这一现象一致。此外，我们还发现，在烟雾暴露幼仔中与粘液细胞（也被称为杯状细胞）分化和与粘液分泌相关的 *SPDEF* 和 *Foxm1* 基因的表达量较高。虽然在新生小鼠的肺中没有杯状细胞的存在，但这一发现可以用于解释之后的较高的杯状细胞发生易感性。

第 3 章的研究延续第 2 章的研究内容，在相同的小鼠抽烟模型中，给予怀孕期间接受香烟烟雾暴露的母鼠的成年后代以烟雾暴露处理。本章旨在探究早期烟雾暴露的后代是否在面对成年期烟雾暴露（3 个月）时其肺组织对炎症和组织修复（重塑）表现得更为敏感。本章中发现的肺组织中发生的变化，和多个信号通路中的基因表达有关，例如一些在炎症、抗氧化系统、肺组织修复和老化过程中起着重要作用的基因，以及将体内的有害物质（香烟烟雾）无害化的相关基因。在本章的研究中，我们发现来自于吸烟母鼠的成年子代，无论它们本身在成年后是否接受烟雾暴露，其体内对炎症、氧化应激和老化的发展有保护作用的相关基因的表达水平都较低，并且与肺组织修复有关的重要基因都有较高的表达水平。

子代自身抽烟会引发组织的重塑（包括平滑肌的增厚更显著，与粘液有关的基因 MUC5AC 的表达水平增加）和炎症（包括对去除烟雾颗粒有重要作用的一种特定类型的白细胞——巨噬细胞的数量增加）。此外，子代成年后接受的烟雾暴露处理也会提高对组织修复和有害物质的解毒起着重要作用的基因的表达水平。值得注意的是，大多数情况下，怀孕期间的早期烟雾暴露并不能引发这些生理学变化。

慢性粘液分泌过剩和慢性阻塞性肺疾病

COPD 是一种无法治愈的、渐进性的肺部疾病，这表现在大气道（慢性气管炎），小气道和肺泡（肺气肿）。暴露于香烟烟雾是罹患 COPD 的主要危险因素。慢性支气管炎与慢性炎性细胞浸润以及过度的气道粘液分泌有关。对于吸烟者和 COPD 患者而言，这种过剩的粘液分泌是产生咳嗽和呼吸困难等症状的重要原因。粘液由在大气道中的杯状细胞

产生，而长期暴露于香烟烟雾会导致杯状细胞数量增加，粘液分泌增多。

在上皮细胞中存在若干信号通路与促进杯状细胞分化及粘液产生有关。格罗宁根哮喘和 COPD 研究中心对吸烟者的研究表明，无肺部疾病的吸烟者及患有 COPD 同时伴有咳嗽和过多粘液分泌症状的吸烟者体内，多种基因在其上皮细胞中具有较高的表达水平。其中一个很重要的基因是 *SPDEF*，该基因是这整个调控网络中的关键基因。已有研究结果表明，SPDEF 可以促进杯状细胞的分化以及粘液的产生。SPDEF 蛋白的表达可以被表观遗传学机制调控，“开启”或“关闭”该基因的表达。

在**第 4 章**中，我们着重针对以下几个问题展开研究：（1）杯状细胞的生长（分化）是否伴随着 *SPDEF* 基因的 DNA 甲基化的变化；（2）比较研究在 COPD 患者和对照组的气道中 *SPDEF* 基因的表观遗传调控的变化。为此，我们从 COPD 患者和对照组的气道中分别分离原代上皮细胞，连续培养四个星期至分化出杯状细胞和纤毛上皮细胞，并且在这个过程中监测基因的表达和 DNA 的甲基化。在这项研究中，我们发现，为促进杯状细胞分化添加信号物质白介素 13（IL-13）后，在对照组上皮细胞的分化过程中伴随着与黏液产生相关的基因如 *MUC5AC* 和 *SPDEF* 表达的增加，以及与抑制黏液产生有关的基因 *FOXA2* 的表达水平的降低。很吸引人的一个实验结果是，该分化过程中 *SPDEF* 基因表达的增加与 *SPDEF* 的 DNA 甲基化的改变之间存在正相关关系。该变化也同样发生在来自于 COPD 个体的上皮细胞分化过程中。此外，在没有加入 IL-13 的培养系统中，我们比较分析了来源于 COPD 个体和对照组个体的上皮细胞分化至第 14 天时相关基因的表达水平和甲基化水平。有趣的是，我们发现，来自 COPD 个体的上皮细胞中 *SPDEF* 和 *MUC5AC* 基因具有较高的表达水平，这与 *SPDEF* 基因的较低的 DNA 甲基化水平有关。由此可知，在 COPD 患者体内 *SPDEF* 基因的表观遗传学机制受到干扰，这也可能是 COPD 患者体内杯状细胞产生和粘液分泌增多的重要原因。

第 5 章主要的研究目的是验证通过改善在前面的章节中发现的表观遗传学变化对 COPD 治疗具有的潜在意义。我们主要想通过增加 *SPDEF* 基因的 DNA 甲基化，从而降低气道中上皮细胞的粘液产生。由于，人肺

细胞系 A549 具有和 COPD 原代细胞类似的 *SPDEF* 和 *MUC5AC* 基因的表达增加和产生黏液等特征，因此在本章中我们选择此细胞系开展相关的研究工作。在本章的研究中，在融合有可与 *SPDEF* 基因特异性结合的 DNA 的结构域（锌指结构）以及 DNA/组蛋白甲基化转移酶的酶活性域的协同作用下，可实现对 *SPDEF* 基因表达的负调节；同时，SPDEF 基因涉及的下游通路中和粘液有关的基因 *MUC5AC* 和 *AGR2* 也被负调控。综上所述，以上研究结果开辟了利用表观遗传学机制（从而可能永久性）地改变 *SPDEF* 基因表达的可能性，这也是一种抑制粘液产生的新的治疗策略。

本文的一般结论

本论文的研究表明，早期（孕期）暴露于香烟烟雾可抑制新生子代小鼠肺中的纤毛细胞的产生，并且可增加与杯状细胞有关的基因 *Spdef* 的表达。在成年小鼠中，早期（孕期）暴露于香烟烟雾对具有不同生理学功能的基因的表达水平有影响，例如可影响与氧化应激，衰老和肺组织修复有关的一些基因的表达。而且，子代在成年期存在的自身的烟雾暴露，对于与炎症，组织重塑，老化以及有毒物质的解毒等过程相关基因的表达有影响。然而这种影响并没有由于孕期的烟雾暴露得到进一步增强。

此外，通过对来源于 COPD 患者的气道上皮细胞的研究，我们推测 *SPDEF* 基因可能在 COPD 的发病机制中起着十分重要的作用，因此该基因可能是一个未来用于治疗 COPD 的良好靶标。由于我们发现在来自 COPD 个体的气道上皮细胞中，*SPDEF* 基因的 DNA 甲基化似乎被干扰，可成为表观遗传学编辑的一个很好的靶标。而且细胞系的初步研究结果也表明，有针对性的增加 *SPDEF* 基因的甲基化确实可抑制 *SPDEF* 基因的表达和粘液的产生。这些极具发展前景的研究成果是通过改变表观遗传学实现 COPD 治疗迈开的第一步，为调节任意靶基因的表达开辟了广阔的前景。但是值得注意的是，进一步的深入研究必须先验证表观遗传学编辑的特异性和长久性，从而最终可实现对机体生理学表现方面的持久的改善（例如抑制粘液分泌）。

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Biography

Juan Song was born on July 29th, 1986 in Anyang, Henan Province, China. From 2005 to 2010, she completed an undergraduate program major in Medical Laboratory Science and minor in law at Tianjin Medical University. In 2010, she was awarded the Bachelor's Degree in Medicine and Law. Subsequently, she started the Combined Master's and Ph.D. Program in the department of Medical Cell biology of Tianjin Medical University, and investigated the role of SND1 in stress granules aggregation and in cell differentiation and proliferation under the supervision of Prof. Jie Yang. In 2013, she was recommended to start her PhD study in the department of Pathology and Medical Biology, University Medical Center Groningen, The Netherlands. Her research project combined both pulmonary pathology (supervised by Dr. M.N. Hylkema) and innovative epigenome editing strategies (in the epigenetic editing group of Prof. Dr. M.G. Rots), which was presented in this thesis entitled "Lung epithelial cell differentiation in human and mouse: environment, epigenetics and epigenetic editing". After her defense of her PhD thesis in May 2016, she intends to continue her academic journey in the field of epigenetics and/or pulmonary disease.

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