Prenatal smoke exposure dysregulates lung epithelial cell differentiation in mouse offspring – Role for AREG-induced EGFR signaling.

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Running Head: Role of AREG in prenatal smoke induced aberrant lung development.
Abstract

Prenatal smoke exposure is a risk factor for impaired lung development in children. Recent studies have indicated that Amphiregulin (AREG), which is a ligand of the epidermal growth factor receptor (EGFR), has a regulatory role in airway epithelial cell differentiation. In this study, we investigated the effect of prenatal smoke exposure on lung epithelial cell differentiation and linked this with AREG-EGFR signaling in 1-day old mouse offspring. Bronchial and alveolar epithelial cell differentiation were assessed by immunohistochemistry. Areg, Egf as well as mRNA expression of specific markers for bronchial and alveolar epithelial cells were assessed by RT-qPCR. Results in neonatal lungs were validated in an AREG-treated 3D mouse lung organoid model. We found that prenatal smoke exposure reduced the number of ciliated cells and the expression of the cilia-related transcription factor Foxj1, whereas it resulted in higher expression of mucus-related transcription factors Spdef and Foxm1 in lung. Moreover, prenatally smoke exposed offspring had higher numbers of alveolar epithelial type II cells (AECII) and lower expression of the AECI-related Pdpn and Gramd2 markers. This was accompanied by higher expression of Areg and lower expression of Egf in prenatal smoke exposed offspring. In bronchial organoids, AREG treatment resulted in fewer ciliated cells and more basal cells when compared non-treated bronchiolar organoids. In alveolar organoids, AREG treatment led to more AECII cells than non-treated AECII cells. Taken together, the observed impaired bronchial and alveolar cell development in prenatally smoke-exposed neonatal offspring may be induced by increased AREG-EGFR signaling.

Keywords: Prenatal smoke exposure; Amphiregulin; Epithelial cell differentiation; Lung organoids.
Introduction

Epidemiological studies have shown that prenatal smoke exposure is associated with asthma in children and adolescents, as well as with chronic obstructive pulmonary disease (COPD) later in life (5, 6, 8, 22, 37). One of the underlying mechanisms could be an aberrant development of the lung, as shown by a number of studies in experimental animal models (9, 14, 28, 30, 31). In these models, prenatal smoke exposure was shown to increase the airway muscle layer (3) and enlarge the airspace size, suggesting an alveolarization defect (14). A similar effect was seen in rhesus monkeys, in which in utero exposure to nicotine resulted in lung hypoplasia and a reduction of the gas-exchange surface in the lung (28).

Epidermal growth factor receptor (EGFR) signaling regulates lung morphogenesis to give rise to the branching of airways and alveolarization (19). In addition, it also has been suggested to be a crucial regulator of goblet cell hyperplasia (33) and mucous hypersecretion (46) in cigarette smoke-exposed airway epithelium. EGFR signaling is mostly induced by two competitive ligands, i.e. amphiregulin (AREG) and epidermal growth factor (EGF) (32, 43). Each ligand has distinct biological functions, i.e. AREG is known to promote cell proliferation, apoptosis, and hyperplasia (7, 17), whereas EGF has a higher affinity to EGFR than AREG and is involved in epithelial cell differentiation (27, 29). AREG is a polypeptide growth factor, which belongs to the EGF family. AREG is expressed by activated immune cells, such as alveolar macrophages (17, 20), mast cells (40), and T helper 2 (Th2) cells (21), and it is regulator as paracrine and autocrine factors in chronic lung inflammation and wound repair. Studies have highlighted AREG-induced EGFR activation through various environmental effects, such as smoking (47), injuries, and infections (20, 39, 45). Recently, Zuo et al. showed that AREG-induced EGFR activation drives airway epithelial cell differentiation towards basal - and mucous cell hyperplasia in an in vitro model of the air-liquid interface (47).

To date, no studies have addressed the role of the AREG-EGFR signaling pathway in prenatally smoke-induced aberrant lung development. With this project, we aimed to investigate the effect of prenatal smoke exposure on lung epithelial cell differentiation (bronchiolar and alveolar) and its link with AREG-induced EGFR signaling in 1-day old mouse offspring.
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 was 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 211± 30 mg/m³ and a CO level of 205± 35 PPM where CO levels of 241 PPM were reached at the 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 gonadotropin 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 formalin and embedded in paraffin for immunohistochemically and immunofluorescence analyses.

qRT-PCR analysis in lung tissue
Total RNA was isolated from lung tissue using an 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 (Mm99999915_g1), Foxj1 (Mm01267279_m1), Scgb1a1 (Cc10, Club cell, Mm00442046_m1), Foxm1 (Mm00514924_m1), Spdef (Mm00600221_m1), (Mm00447558_m1), Aqp5 (Mm00437578_m1), Gramd2 (Mm01174485_m1), Nkx2.1 (Mm00447558_m1), Egf
Expression Assays were used (T, 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-ΔCp (Cp means crossing points). Undetectable Cp values of the genes of interest (>40) were interpreted as the maximum Cp value.

**Lung organoids culture**

Mouse lung organoids were grown as described previously (16, 24). Briefly, ~10,000 EpCAM+ mouse lung cells were seeded with an equal number of fibroblasts per insert in 100μl growth factor-reduced Matrigel, diluted 1:1 with DMEM/F12 containing 5% FBS into transwell inserts for 24-well. In the treated lung organoids groups, AREG (10ng/ml; R&D System, Minneapolis, USA) was freshly added to the lung organoids every 2 days. All mouse lung organoid cultures were maintained at 37°C in a humidified incubator with 5% CO2. After 14 days, lung organoids were fixed in acetone that was diluted 1:1 (v/v) with methanol for 15 min at -20°C. For bronchial epithelial cell analyses, organoids were prepared for the histology of cross-sectional membranes, as published previously (2). The histology of cross-sectional membranes was stabilized in 2% agarose dehydrated, and paraffin-embedded. Organoids were stained for basal cells (KRT5), club cells (CC10), ciliated cells (tubulin), AECI (PDPN) and AECII (pro-SPC) All used antibodies are listed in Table 1.

**Immunohistochemistry (IHC) and Immunofluorescence (IF) staining**

**Lung tissue**

Sections (3 μm) of formalin-fixed and paraffin-embedded lung tissue were stained for ciliated cell, club cells, AECI and AECII using standard IHC method. Briefly, slides were deparaffinized and put in citrate (pH 6.0) and Tris-HCl (pH 7.4) buffer in a microwave oven for 15min. After cooling, slides were incubated in 0.3% H2O2 in PBS for 30 min. To visualize ciliated cells (Tubulin), club cells (CC10), AECII (pro-SPC and NKX2.1), AECI (PDPN), and AREG-expressing cells, they were incubated with the specific primary antibodies, as listed in Table 1. As a second step, binding of primary antibodies was detected by using enzyme horseradish peroxidase (HRP) and the biotinylated conjugated secondary antibodies for 30 min. For the color reaction, the secondary antibodies were detected by A 0.05% diaminobenzidine (DAB, Sigma-Aldrich, The Netherlands) and
3-amino-9-ethylcarbazole (AEC, Sigma Aldrich) solutions. Numbers of tubulin and CC10
positive cells in all airways were counted manually, and total the number of CC10 and tubulin
positive cells were corrected for per mm airway. These analyses were assessed by using
Aperio-ImageScope viewing software (Aperio, 11.2.0.780, Vista, USA). Numbers of pro-
SPC, NKX2.1, AREG and PDPN positive cells were analyzed by using morphometric
analysis in Aperio-ImageScope viewing software, and corrected for the total area of the lung
tissue per section.

Lung organoids

Paraffin-embedded organoids were stained double or triple stained with primary antibodies
against KRT5 (basal cell), CC10 (club cell) and tubulin (Ciliated cell). The expression of
these markers was visualized by staining with fluorescent secondary antibodies and the
mounting medium containing DAPI (Abcam, Cambridge, UK). Immunofluorescence (IF)
slides were scanned using a TissueFaxs imaging system (Tissue Gnostics, Austria), and
images were acquired using a Leica SP8 (Leica) confocal and a Leica 4000b microscopes at
40x magnification. For alveolar organoids, organoid cultures were washed with PBS and
blocked in PBS with 5% (w/v) BSA. Primary antibodies against pro-SPC (AECII) and PDPN
(AECI) were diluted in PBS buffer with 0.1% BSA and 0.1% Triton, and the organoids
cultures were incubated with primary antibodies at 4°C overnight. The next day, the organoids
were washed with PBS for 30 min, and AECI and AECII were detected using fluorescent
secondary antibodies and the mounting medium containing DAPI. The alveolar organoids’
images were acquired using the Leica SP8 confocal microscope at 40x magnification.
Processed channels were merged or split using ImageJ software. Positive cells were counted
manually and positive cell numbers were corrected for the total number cells of bronchial and
alveolar epithelial cells in mouse lung organoids.

Statistical methods

For statistical evaluation of the different groups, a two-tailed Mann-Whitney U-test was used
(GraphPad Prism 7.02 Software, San Diageo, CA and SPSS Statistics 23, IBM, The
Netherlands). Differences between the prenatal smoke-exposed offspring and controls were
assessed separately in males and females with a two-sided Mann-Whitney U-test. A value of
p<0.05 was considered significant. Relative gene expression (2^ΔCt method) and standard error
of the mean (SEM) were calculated in Microsoft® Office Excel 2016.
Results

To investigate how prenatal smoke exposure affects on lung development, we began by exploring the bronchial epithelial cell development, which was visualized by IHC analyses of tubulin-expressing (ciliated) cells and CC10-expressing (club) cells, as shown in Figures 1A and C. Prenatally smoke-exposed neonates had lower numbers of ciliated cells when compared to the controls (p< 0.001; Figure 1B), which was observed in both male and female offspring (p< 0.0001; Figure 1b). This was accompanied by lower expression of Foxj1, especially in prenatally smoke-exposed male offspring (p= 0.03; Figure 1E, male: p=0.04; Figure 1e). In contrast, no effect of prenatal smoke exposure was observed on the number of club cells (Figure 1D and 1d) or expression of Scgb1a1 mRNA levels (club cell marker) in both groups (data not shown). Prenatally smoke-exposed neonates did have higher mRNA expression of other key players in secretory cell formation such as Foxm1 (p=0.014, Figure 1F, male: p=0.01, female: p=0.04; Figure 1f) and Spdef (p<0.001; Figure 1G, male: p<0.001; Figure 1g).

For alveolar epithelial cell development, we asked whether AECII (Figures 2A and C) and AECI (Figure 3A) differentiation was affected by prenatal smoke exposure in this mouse model. Prenatally smoke-exposed offspring had a higher number of pro-SPC (AECII) and NKX2.1 (AECII)-positive cells in both sexes compared to their control groups (p=0.001; Figure 2B, both sexes: p<0.05; Figure 2b and p=0.0005; Figure 2D, male: p=0.05, female: p=0.006; Figure 2d). In contrast, the number of PDPN positive (AECI) cells was not affected by prenatal smoke exposure (Figure 3B and 3b) but lower mRNA levels of the Pdpn and Gramd2 were observed in prenatally smoke-exposed offspring when compared to the control group (p<0.0001; Figure 3C, male: p=0.04, female: p=0.006; Figure 3c and p<0.0001, Figure 3E, both sexes: p<0.001; Figure 3e). Aqp5 mRNA levels were not affected by prenatal smoke exposure (Figure 3D and 3d).

We next investigated the role of AREG in the prenatal smoke-induced aberrant cell differentiation. First, we identified AREG-expressing cells, which could be mainly observed in the lung parenchyma, as shown in Figure 4A. A higher number of AREG-expressing cells was found in prenatally smoke-exposed offspring when compared to the control group (p =0.04, Figure 4B), which was most pronounced in females (p =0.012, Figure 4b). Furthermore, higher Areg mRNA levels were found in prenatally smoke-exposed offspring when compared to the control group in both sexes (p=0.0014, Figure 4C; both sexes: p<0.05, Figure 4c). In contrast, lower Egf mRNA levels were detected in prenatally smoke-exposed
offspring (p=0.0018, Figure 4D, female: p=0.02; Figure 4d) when compared to the control group. *Egfr* mRNA levels were not affected by prenatal smoke exposure (Figure 4E and 4e).

Based on these results, we next asked whether indeed AREG was able to affect lung epithelial cell differentiation. To answer this question, we used a 3D mouse lung organoid model and compared epithelial cell differentiation in AREG-treated and non-treated lung organoids. Epithelial cell differentiation in these mouse lung organoids was assessed 14 days after treatment as shown in Figure 5A. In bronchiolar organoids, ciliated, club and basal cells were visualized in cross-section slides using triple IF staining for tubulin, CC10 and KRT5, as shown in Figure 5B. After counting whole organoids, we found lower numbers of tubulin-positive cells (p<0.001, Figure 5D) and higher numbers of KRT5 positive cells (p=0.02, Figure 5C) in AREG-treated lung organoids when compared to non-treated lung organoids. AREG treatment did not affect the number of CC10-positive cells in bronchiolar organoids (Figure 5E). Furthermore, we assessed alveolar epithelial cell differentiation for which pro-SPC (AECII) and PDPN (AECI)-positive cells (Figure 5F) were counted in AREG-treated and non-treated groups. More pro-SPC positive cells were detected in AREG-treated alveolar organoids when compared to non-treated alveolar organoids (p=0.03, Figure 5G), while no effect was seen for the number of PDPN-positive cells (Figure 5H).
Discussion

In the present study, we evaluated the adverse effect of maternal smoking during pregnancy on lung epithelial cell development in neonatal offspring, and investigated the possible role of AREG on epithelial development in this process. With respect to bronchial epithelial cells, prenatal smoke exposure resulted in fewer ciliated cells and downregulation of Foxj1, which was accompanied by higher expression of secretory transcription factors Spdef and Foxm1. Regarding alveolar development, a higher number of AECII was found, whereas lower Pdpn and Gramd2 mRNA levels were observed in prenatally smoke-exposed neonates. These epithelial cell alterations may be mediated through AREG-induced EGFR signaling, as in prenatally smoke-exposed offspring higher numbers of AREG-expressing cells were found and higher Areg mRNA levels were accompanied by a lower expression of Egf. A possible role of AREG in epithelial cell differentiation was confirmed in mouse lung organoids, showing that AREG suppressed the number of ciliated and increased the number of AECII.

Ciliated cells are necessary for proper mucociliary clearance of particles and pathogens (41), and cilia dysfunction or a decrease of ciliated cell numbers may account for repeated chest infections and tissue destruction in the lung (36). Indeed, epidemiological studies have shown that children born from a mother that smoked during pregnancy have a higher risk to suffer from asthma and airway infections (4, 44). Our data is also supported by a previous in vitro study in which primary human bronchial epithelial cells were exposed to cigarette smoke extract during differentiation at the air-liquid interface (26). In that study, cigarette smoke extract exposure was shown to reduce the number of ciliated cells, while it increased the number of club cells and goblet cells. In our study, lower numbers of ciliated cells were accompanied by lower expression of Foxj1. In mice, club cells are thought to be progenitors of ciliated cells and goblet cells (34, 35) which are the two major cell types in the proximal airways of the mouse. Therefore, more club cells in prenatally smoke-exposed offspring were expected. However, the lower numbers of ciliated cells were not accompanied by the presence of more CC10-positive club cells in our mice, which may be due to a large variation in club cell numbers between the different mice. However, prenatal smoke exposure did increase the expression of Foxm1 and Spdef. Foxm1, a transcription factor of the Forkhead box family, plays an important role during embryonic development of alveolar surfactant homeostasis and monocyte/macrophage recruitment (10). Moreover, FOXM1 is known to promote SPDEF activity, a master transcription factor that regulates goblet cell differentiation in the airway epithelium (42). Therefore, higher expression of Foxm1 could have contributed to the higher expression of Spdef in offspring from smoke-exposed mothers that we found.
We next assessed the distribution of alveolar epithelial cells in our model. In the current study, prenatal smoke exposure increased the number of AECII, which was accompanied by lower *Pdpn* and *Gramd2* mRNA expression in 1-day-old mouse offspring. Similarly, studies have shown that AECII proliferate to restore the alveolar epithelium in response to cigarette smoke-induced lung injuries, whereas AECI are more susceptible to die from the same exposure (12, 18). In our study, however, the number of AECI was not different in prenatal smoke-exposed mice compared to controls. A similar effect was seen in prenatal nicotine-exposed rat offspring (25). In that study, intrauterine exposure to nicotine increased AECII proliferation and altered the metabolic activity of surfactant synthesis in AECII. Furthermore, in rhesus monkeys, intrauterine nicotine exposure resulted in a higher number of AECII and a higher mean linear intercept of airspaces, which was accompanied by a reduced alveolar complexity in the lung (28). These models all show a preference for AECII self-renewal after injury induced by a intrauterine exposure. In the case this preference persists in the alveolarization stage, it could impair differentiation from AECII to AECI after injury and limit the regenerative capacity of the lung later in life.

Our results are also in line with human epidemiological studies, showing that prenatal smoke exposure is associated with deficits of the functional residual capacity of the lung and tidal flow volumes (1, 11), which could be a high risk for an insufficient gas exchange in prenatally smoke-exposed infants.

Although prior reports have described that AREG promotes lung fibrosis (7), and mucous cell metaplasia in the airway (15), the role of AREG in lung development is not understood. To our knowledge, this is the first study showing that prenatal smoke exposure increased the number of AREG-expressing cells in the lung. This result is consistent with human studies in the lung, showing that urban fine particulate matter (38) and cigarette smoke (47) can stimulate AREG mRNA expression. In addition, AREG was shown to be increased in sputum samples from children with asthma (13). The implications of more AREG-expressing cells in our model could be that AREG, as a paracrine factor, promoted *Spdef* expression, a required regulator for club cell and goblet cell differentiation (15), which explains the reduced ciliated cell number in prenatal smoke exposed offspring. This is consistent with a study by Zuo et al. where they assessed the role of EGFR signaling in human airway basal cell differentiation upon AREG and EGF treatment in an air-liquid interface cell model and found that AREG-induced EGFR signaling promoted mucous cell hyperplasia and suppressed ciliated cell differentiation (47). Another study showed that EGF-induced EGFR activation altered the morphology of airway basal cells with the loss of airway
intercellular contacts (29). Interestingly, in our model, prenatally smoke-exposed offspring had higher *Areg* mRNA levels which was accompanied by lower *Egf* mRNA levels. These results could implicate that the relative lack of EGF supports the binding of AREG to EGFR, for which the receptor normally has a lower affinity compared to EGF (43).

AREG has been described in lung epithelial and mesenchymal cells from embryonic mice (27), where it stimulates epithelial and mesenchymal cell proliferation, particularly at the tips of growing lung buds. This suggests that the location of AREG expressing cells and its target cells are an important factor in determining regulation of AREG. In the current study, our findings further elaborate on the unrecognized role of a cross-talk between AREG expressing cells and AECII responder cells regarding differentiation and proliferation, which might have a negative effect on the regenerative capacity of the lung later in life.

To validate our data obtained in the prenatally smoke-exposed model, and to further investigate the direct effects of AREG on epithelial cell differentiation, we used a 3D mouse lung organoid model. In this model, isolated AECII cells were cultured with fibroblasts for 14 days until lung organoids were formed (24). From Day 1 on, they were treated with AREG protein or PBS, every other day for 14 days. We found more basal cells and lower ciliated cell numbers in AREG-treated lung organoids, suggesting that AREG selectively supports the proliferation of basal cells, which may be at the expense of ciliated cell differentiation. This result supports both our results in prenatal smoke exposed offspring as it supports the study from Zuo *et al*, in the 2D air liquid interface cultured AREG-treated airway epithelium (47).

To elaborate on the role of AREG in AECII differentiation in our organoid model, it is of interest to note that AREG is upregulated in lung fibrosis and linked with lung fibroblast proliferation (7). Because AREG can promote fibroblast proliferation, it is possible that AREG induces fibroblast activation in our AREG-treated lung organoids, which might have a negative impact on epithelial cell differentiation during lung organoid formation. Indeed, the efficiency of lung organoid-forming was shown to be reduced by TGF-β pre-treatment of fibroblasts in the same model (23). However, further studies are needed to determine the effect of AREG on fibroblast activation and lung epithelial cell differentiation in lung organoids.

Next to our results in the bronchial organoids, a higher number of AECII cells was observed in AREG-treated alveolar lung organoids, whereas the number of AECI was not altered. This observation regarding impaired AECII stem cell function fits with the earlier described stimulating effect of AREG on basal cell numbers, which are important for airway wall regeneration. Therefore, more studies are needed to further investigate the impact of
To conclude, we provide evidence that prenatal smoke exposure impairs lung development in the offspring in which increased AREG/EGFR signaling may have a role. During bronchiolar development, prenatal smoke exposure resulted in fewer ciliated cells and lower expression of cilia-related transcription factor of Foxj1, whereas it promoted secretory cell differentiation through regulation of Foxm1 and Spdef. During alveolar development, prenatal smoke exposure resulted in more AECII which was accompanied by lower expression of AECI-related markers Pdpn and Gramd2, which may cause a delay in alveolar development. As the AREG/EGFR signaling pathway relates to impaired epithelial cell differentiation, AREG regulation during lung development may provide a novel intervention for aberrant lung development in children from smoking mothers.

Abbreviations: Alveolar epithelial cell (AEC), chronic obstructive pulmonary disease (COPD), amphiregulin (AREG),

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Disclosure

The authors declare they have no competing financial interests.

Author contribution


Reference


18. Messier EM, Bahmed K, Tuder RM, Chu HW, Bowler RP, Kosmider B. Trolox contributes to Nrf2-mediated protection of human and murine primary alveolar type II


35. Tata PR, Mou H, Pardo-Saganta A, Zhao R, Prabhu M, Law BM, Vinarsky V,


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Figure 1. Ciliated cell (A) and club cell (C) were stained by tubulin and CC10 markers in paraffin-embedded slide with DAB (brown) substrate. The number of tubulin+ (B and b) and CC10+ (D and d) cells which were determined in lung tissue prenatally exposed to cigarette smoke or not, original magnification 40x. E and e present gene expression of Foxj1, a marker for ciliated cells the differentiation markers. Genes that drive secretory cell development, gene expression of the transcription factor Foxm1 (F and f), and Spdef (G and g) were analyzed by real-time qPCR in RNA, isolated from lung tissue. A lowercase letter indicates a sex-dependent (male, female) difference in the data. Data represent means of expression in offspring of air-exposed mothers (open symbols) or smoke-exposed mothers (closed symbols). Circle (○) symbol(s)=male, triangle (Δ) symbol(s)=female. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. *p≤0.05, **p≤0.01, ***p≤0.001 (Mann-Whitney U-test).

Figure 2. AECII cells were stained by pro-SPC (A) and Nkx2.1 (C) markers in paraffin-embedded slide with DAB (brown) substrate, original magnification 40x. The number of SPC+ (B and b) and Nkx2.1+ (D and d) cells significantly increased in neonatal offspring from smoke-exposed mothers. A lowercase letter indicates a sex-dependent (male, female) difference in the data. Data represent means of expression in offspring of air-exposed mothers (open symbols) or smoke-exposed mothers (closed symbols). Circle (○) symbol(s)=male, triangle (Δ) symbol(s)=female. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. *p≤0.05, **p≤0.01, ***p≤0.001 (Mann-Whitney U-test).

Figure 3. The number of PDPN+ cells (B and b) was quantified in a paraffin-embedded slide with DAB (brown, A) substrate, original magnification 40x. The Pdpn (C and c) Aqp5 (D and d) and Gramd2 (E and e) mRNA levels were analyzed by real-time qPCR in isolated RNA from lung tissue. A lowercase letter indicates a sex-dependent (male, female) difference in the data. Data represent means of expression in offspring of air-exposed mothers (open symbols) or smoke-exposed mothers (closed symbols). Circle (○) symbol(s)=male, triangle (Δ) symbol(s)=female. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. *p≤0.05, **p≤0.01, ***p≤0.001 (Mann-Whitney U-test).
Figure 4. A presents IHC staining of AREG-expressing cells (red). The number of AREG-expressing cells (B and b) was quantified in lung tissue prenatally exposed to cigarette smoke or not, original magnification 40x. The red arrow shows AREG-expressing cells in lung tissue. Gene expression levels of Areg (C and c), Egf (D and d) and Egfr (E and e) were analyzed by real-time qPCR in RNA, isolated from lung tissue. A lowercase letter indicates a sex-dependent (male, female) difference in the data. Data represent means of expression in offspring of air-exposed mothers (open symbols) or smoke-exposed mothers (closed symbols). Circle (○) symbol(s)=male, triangle (△) symbol(s)=female. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. *p≤0.05, **p≤0.01 (Mann-Whitney U-test).

Figure 5. 3D mouse organoids formation and lung epithelial cell differentiation in AREG-treated and non-treated groups. Representative light and confocal microscopes pictures show mouse lung organoids formation at 14 days (A, AREG-treated organoids). Original magnification 20x or 40x. The representative picture shows triple IF staining (B) in bronchiolar organoids and double IF staining (F) in alveolar organoids. The number of Tubulin⁺ (C, green cells), CC10⁺ (D, yellow cells) and KRT5⁺ (E, red cells) were in bronchiolar mouse organoids. Quantification analysis of pro-SPC⁺ (G) and PDPN⁺ (H) was performed in alveolar mouse organoids. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. *p≤0.05, *p≤0.001 (Mann-Whitney U-test).
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Figure 1

(A) air-exposed

(B) Ciliated + cells (μm airway)

(C) smoke-exposed

(D) CC10+ cells (μm airway)

(E, e) Fos (Z-

(F, f) Foxm1 (Z-

(G, g) Spdef (Z-

[Additional details and explanations may be necessary to fully understand the figure's content.]
Figure 3

A. Air-exposed vs. smoke-exposed Pdpn expression.

B. Distribution of PDPN+ cells (per μm²) for different conditions.

C. Pdpn expression levels for male and female subjects under air and smoke exposure.

D. Aqp5 expression levels for male and female subjects under air and smoke exposure.

E. Gramd2 expression levels for male and female subjects under air and smoke exposure.
Figure 4

A) air-exposed vs. smoke-exposed AREG expression

B) Scatter plots showing AREG+ cells per μm² for different conditions

C) Graphs comparing Areg (Z-score) and Egrf (Z-score) expression in air and smoke conditions for males and females

D) Graphs showing Areg and Egrf expression levels in air and smoke conditions for males

E) Graphs showing Areg and Egrf expression levels in air and smoke conditions for females
Figure 5
F  control lung organoids  AREG-treated lung organoids

G  %ProSP-C⁺ cells (alveolar organoids)
H  %PDNP⁺ cells (alveolar organoids)