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**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.

30 **Abstract**

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

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51 **Keywords:** Prenatal smoke exposure; Amphiregulin; Epithelial cell differentiation; Lung
52 organoids.

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60 **Introduction**

61 Epidemiological studies have shown that prenatal smoke exposure is associated with asthma
62 in children and adolescents, as well as with chronic obstructive pulmonary disease (COPD)
63 later in life (5, 6, 8, 22, 37). One of the underlying mechanisms could be an aberrant
64 development of the lung, as shown by a number of studies in experimental animal models (9,
65 14, 28, 30, 31). In these models, prenatal smoke exposure was shown to increase the airway
66 muscle layer (3) and enlarge the airspace size, suggesting an alveolarization defect (14). A
67 similar effect was seen in rhesus monkeys, in which *in utero* exposure to nicotine resulted in
68 lung hypoplasia and a reduction of the gas-exchange surface in the lung (28).

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

85 To date, no studies have addressed the role of the AREG-EGFR signaling pathway in
86 prenatally smoke-induced aberrant lung development. With this project, we aimed to
87 investigate the effect of prenatal smoke exposure on lung epithelial cell differentiation
88 (bronchiolar and alveolar) and its link with AREG-induced EGFR signaling in 1-day old
89 mouse offspring.

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93 **Material and methods**

94 **Animals**

95 Female and male C57Bl/6 mice, age 8-10 weeks, were obtained from Harlan (Horst, The
96 Netherlands). Mice had access to standard food and water ad libitum. The animal study was
97 approved by the Institutional Animal Care and Use Committee of the University of Groningen
98 (Permit Number: 6589a) and was performed under strict governmental and international
99 guidelines on animal experimentation.

100 **Cigarette smoke exposure**

101 Mainstream cigarette smoke was generated using a TE-10 smoke exposure system of Teague
102 Enterprises Smoke Exposure System (Woodland, California, USA). Female mice were
103 exposed to fresh air (n=3) or cigarette smoke (n=4) in two sessions of 50 minutes with a 3h
104 interval between both exposures per day in which smoke of 10 cigarettes was generated per
105 session. Mice were exposed from 7 days before mating until the day of sacrifice. The adaption
106 protocol included exposure to 3 cigarettes per session the first day, 5 cigarettes the second
107 day, 7 cigarettes the third day and 10 cigarettes the fourth day and thereafter. Smoking 10
108 cigarettes in one session generated total particulate matter counts of 211 ± 30 mg/m³ and a CO
109 level of 205 ± 35 PPM where CO levels of 241 PPM were reached at the max. Kentucky
110 2R4F research-reference filtered cigarettes (The Tobacco Research Institute, University of
111 Kentucky, Lexington, Kentucky, USA) were used. For experimental purposes, female mice
112 were treated with 1.25 IU pregnant mare's serum gonadotropin and 1.25 IU human chorionic
113 gonadotrophin to induce simultaneous cycling. To induce pregnancy, 1 female was housed
114 with 1 male. Mating was confirmed by vaginal plug detection. Smoke exposure remained
115 constant during the total pregnancy. Mothers and offspring were not exposed to cigarette
116 smoke after offspring was born. Offspring (n=16 from non-smoking mothers, n=24 from
117 smoking mothers) was sacrificed one day after birth. The left lung was used for qRT-PCR
118 analyses. The right lung was fixed in formalin and embedded in paraffin for
119 immunohistochemically and immunofluorescence analyses.

120 **qRT-PCR analysis in lung tissue**

121 Total RNA was isolated from lung tissue using an RNA isolation trizol kit (Invitrogen, USA).
122 cDNA was reverse transcribed using a Superscript-II Reverse Transcriptase kit (Invitrogen,
123 USA). To measure the expression of *Gapdh* (Mm99999915_g1), *Foxj1* (Mm01267279_m1),
124 *Scgb1a1* (Cc10, Club cell, Mm00442046_m1), *Foxm1* (Mm00514924_m1), *Spdef*
125 (Mm00600221_m1), (Mm00484714_m1), *Ppdn* (Mm00447558_m1), *Aqp5*
126 (Mm00437578_m1), *Gramd2* (Mm01174485_m1), *Nkx2.1* (Mm00447558_m1), *Egf*

(Mm00438696_m1), *Areg* (Mm 01354339_m1), *Egfr* (Mm01187858_m1), on demand Gene Expression Assays were used (T, USA). PCR reactions were performed in triplicate in a volume of 10 μ L consisting of 2 μ L of MilliQ water, 5 μ L PCR master mix (Eurogentec, Seraing, Belgium), 0.5 μ L assay mix (life technologies, USA), and 2.5 μ L 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 $2^{-\Delta C_p}$ (C_p means crossing points). Undetectable C_p values of the genes of interest (>40) were interpreted as the maximum C_p value.

Lung organoids culture

Mouse lung organoids were grown as described previously (16, 24). Briefly, $\sim 10,000$ EpCAM⁺ mouse lung cells were seeded with an equal number of fibroblasts per insert in 100 μ L 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 37⁰C 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 -20⁰C. 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 μ m) 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 4⁰C 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 ($2^{-\Delta Ct}$ 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 *Gramp2* 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 an 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

AREG/EGF-induced EGFR signaling on basal-and alveolar epithelial cell proliferation, remodeling and regeneration in the lung.

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

L.K., S.S. S.K., J.C. and M.N.H. conceived and designed research; L.K., Z.Z. J.S., S.S, M.R, W.K., and J.C. performed experiments; L.K. Z.Z., S.S, J.C. analyzed data; L.K. Z.Z. J.S., S.S, M.R, W.K., BNM and J.C interpreted results of experiments; L.K. and J.C. prepared figures; L.K. J.A.P. and T.M.N. drafted manuscript; L.K., S.K., J.C., BNM and M.N.H. edited and revised manuscript; L.K. Z.Z. J.S., S.S, M.R, W.K., S.K. J.C, BNM and M.N.H. approved the final version of manuscript.

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542

543 FIGURE CAPTIONS

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

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

565 **Figure 3.** The number of PDPN⁺ cells (B and b) was quantified in a paraffin-embedded slide
 566 with DAB (brown, A) substrate, original magnification 40x. The *Pdpn* (C and c) *Aqp5* (D and
 567 d) and *Gramd2* (E and e) mRNA levels were analyzed by real-time qPCR in isolated RNA
 568 from lung tissue. A lowercase letter indicates a sex-dependent (male, female) difference in the
 569 data. Data represent means of expression in offspring of air-exposed mothers (open symbols)
 570 or smoke-exposed mothers (closed symbols). Circle (○) symbol(s)=male, triangle (Δ)
 571 symbol(s)=female. Data are shown as individual values. If not stated otherwise, the
 572 difference between groups was not significant. *p≤0.05, **p≤0.01, ***p≤0.001 (Mann-
 573 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 (Δ) symbol(s)=female. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. * $p \leq 0.05$, ** $p \leq 0.01$ (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. * $p \leq 0.05$, * $p \leq 0.001$ (Mann-Whitney U-test).

Antigen	Host and binder recognition	Dilution	Company
Primary antibody			
Tubulin	Mouse, anti-acetylated a-tubulin	1:10000 IHC	Sigma-Aldrich, T7451
CC10	Rabbit, anti-club cell (10 kD)	1:6000 IHC	Millipore, 07-623
CC10	Goat, anti-club cell	1:200 IF	Santa-Gruz, sc-365992
ProSP-C	Rabbit, anti-SP-C	1:200 IHC	Millipore, AB3786
Pdpn	Hamster, anti-Podoplanin	1:5000 IHC, IF	Thermo Scientific, 14-5381-82
KRT5	Chicken, anti- Keratin 5	1:200 IHC, IF	Biologend, 905901
Nkx2.1	Mouse, anti- Nkx2.1	1:200 IHC, IF	Thermo Scientific, MA5-13961
Amphiregulin	Rabbit, anti-amphiregulin	1:100 IHC, IF	Boster, A01787
Amphiregulin	Rabbit, anti-amphiregulin	1:50 IF	Biorbyt, orb4539
Secondary antibody			
Rabbit	Anti-goat HRP	1:100 IHC	Dako, P0449
Goat	Anti-rabbit HRP	1:100 IHC	Dako, P0448
Goat	Anti-hamster HRP	1:200 IHC	Southern Biotech, 6061-05
Rabbit	Anti-Goat BIOT	1:100 IHC	Southern Biotech, 6160-08
	Streptavidin-HRP	1:300 IHC	Dako, P0397
Rabbit	Anti-rat HRP	1:100 IHC	Dako, P0450
Donkey	Anti-goat Alexa Fluor 488	1:100 IF	Thermo Scientific, A11055
Donkey	Anti-rabbit Alexa Fluor 488	1:100 IF	Thermo Scientific, A21206
	Streptavidin, Alexa Fluor 555	1:300 IF	Thermo Scientific, S21381

Figure 1

Figure2

Figure 3

Figure 4

Figure 5

A

B

control lung organoids

AREG-treated lung organoids

F

control lung organoids

AREG-treated lung organoids

G **H**