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Smoking during pregnancy and prenatal programming

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Chapter 8:

Summary/General discussion/Future perspectives

Summary

The scope of this thesis was to investigate the programming effects of prenatal smoke exposure (PSE). Throughout this thesis, offspring from two different mouse models of maternal smoking during pregnancy were used and DNA methylation was assessed in lung and liver, as well as bronchial, tracheal and esophageal epithelium. In combination with the assessment of gene expression, we tried to establish a causal link between aberrant promoter methylation and deregulated mRNA levels. **Chapter 2** shows that PSE-induced changes to promoter methylation patterns are detectable in lungs at thirty days after birth. Moreover, it suggests that the observed effects are sex-specific, stressing the increased susceptibility of females. **Chapter 3** reveals that the in chapter 2 described programming effects are gene-specific and that, again, its extent is more pronounced in female than in male mice. Moreover, the effect of PSE on DNA methylation and gene expression of *Cyp2a5* that is relevant for xenobiotic metabolism are discussed in **Chapter 4A**. It shows in accordance with the analyses above, that PSE can alter the sex-dependent methylation profile of a gene and shows once more reduced gene expression in female mice. **Chapter 4B** extends the assessment of chapter 4A, demonstrating in a second mouse model distinct methylation profiles of *Cyp2a5* in lung and liver, but also organ-specific effects of PSE. **Chapter 5** elaborates on organ-specific promoter methylation patterns in fetal and three-day-old mouse offspring, investigating *Igf1* and *Igf1r*. The naturally established methylation profiles are affected by PSE, dependent on the CpG-site, and the offspring's sex. The comparison of fetal and neonatal data suggests that even late-term gestation is affected by cigarette smoke, but it also indicates that the cellular composition of organs or rapid proliferation of a specific cell type may influence the analysis' outcome. **Chapter 6** follows up on this observation and illustrates differentially methylated gene profiles in bronchial, tracheal and esophageal epithelium. **Chapter 7** provides a review of inter- and transgenerational effects of smoking during pregnancy.

General discussion

(Re)programming

The fetal but also the early postnatal development mark early critical life stages of high vulnerability as e.g., the xenobiotic detoxification as well as the respiratory or immune system are still in the process of development[1,2]. Consequently, environmental insults, such as smoking during pregnancy bear potential to influence those stages, hence affecting the offspring's (future) health[3].

In recent years, the concept of (epi)genomic alterations, especially aberrant DNA methylation, as a possibly relevant link between the *in utero*/early life environment and risk for disease development later in life received a lot of attention. Indeed, global and site-specific aberrant DNA methylation were found in prenatally smoke exposed children[4-7] and the persistence of CpG-site specific differences in DNA methylation states was comparaed at birth, seven years, and 17 years of age[8].

By using two different mouse models on smoking during pregnancy, we showed throughout this dissertation, that PSE induces alterations to promoter methylation profiles. The described changes are CpG-site specific, and vary when comparing both male and female offspring (Chapters 2-6), different organs (Chapter 4B & 5) or tissue dissects of specific cellular compartments (Chapter 6), or different time points (Chapter 4B & 5). Moreover, we show that modeling pregnancy smoking by both, whole-body or nose-only exposure, leads to disturbances on the fetal site.

While the described associations between complete (de)methylation and a gene 'being switched on or off' account for large differences in methylation levels, a new paradigm focuses on small DNA methylation changes. Those are often seen to be caused by environmental influences, at limited genomic regions or CpG-site specific and during period of epigenetic sensitivity (reviewed in ref.[9]). Following this model, these small methylation differences, similar to our findings within Chapters 2-6, often are only 1-5% but are sufficient to disturb the "fine-tuning" of a gene's methylation signature and thereby become the hallmark of non-malignant disorders. These small methylation changes are also found in analyses of newborns from smoking mothers[6,10] and were described e.g., for allergic asthma in adults and children[11]. Although differences in DNA methylation was first thought to alter transcriptional activity that, in turn, correlates with disease, it becomes more and more clear that also other epigenetic factors may be involved. Altered histone marks, deregulated microRNA expression patterns, or chromatin reorganization may all play a role in prenatal programming, and were shown to be affected by (prenatal) smoke exposure[12-14].

Sex differences

Sex-specific effects of PSE were shown for e.g., fetal growth[15,16] or hepatic gene expression[17,18]. The latter observation matches the earlier report upon smoke exposure-mediated increase of DNA methyltransferase expression in liver. This effect seemed more pronounced in female than in male liver, but did not reach statistical significance[19]. Moreover, also prenatal short- and long-term exposure to fine particulate matter induced sex-dependent gene expression[20].

As indicated throughout this dissertation, the effect of smoking during pregnancy on gene promoter methylation differs when comparing male and female mice (Chapters 2 - 6). Within the study that was conducted by Murphy and colleagues[6], the DNA methylation status in umbilical cord blood was assessed and compared between infants from smokers and non-smokers. They found hypermethylation at the IGF2 locus in the smoke-exposed group and that this effect was most pronounced in male offspring[6]. More recently, a postnatal pathway was described in liver that caused demethylation. It occurred exclusively in males, as demethylation appeared to be mediated by the secretion of testosterone and was found to take place at tissue-specific enhancer sequences that in turn affected "transcriptional regulation of nearby genes" [21]. Different base line expression levels of DNA methyltransferases were found when comparing human male and female fetal liver. These sex-differences were lost after prenatal smoke exposure[22]. Similarly, also IGF2 gene expression was sex-dependent and only in male fetuses a reduced expression due to PSE was reported. Additional methylation analysis showed different base line methylation that was also affected by PSE, dependent on the analyzed region and the offspring's sex[22].

Organ differences

We highlighted organ-specific gene promoter methylation profiles that are disturbed by PSE in chapter 4B and 5. As discussed there, genome-wide association studies in human blood samples are becoming increasingly common, as blood samples are the easiest obtainable tissue but also as an experimental setting such as we applied for our study would simply be not feasible in human context. However, we indicated that the cellular composition of both lung and liver may be a confounding factor, especially when comparing fetal and neonatal methylation profiles. It was demonstrated by Jaffe and colleagues that indeed cell composition of peripheral blood tissue accounts for a considerable confounder that explains a huge variability in DNA methylation data[23]. Furthermore, gestational exposure to air pollution was shown to influence the subpopulation of cord blood lymphocytes[24]. These observations together with our findings stress the limited significance of these genome-wide association studies, if those aim to display smoking-associated disturbances in the fetus itself. They also emphasize the importance of accurate DNA methylation analysis, as all organs and dissected tissues are of heterogeneous nature, containing different cell lineages at different differentiation or maturity stages.

One possible way to assess DNA methylation in tissues of multiple cell compounds is the application of cell isolation techniques, as for instance laser capture micro-dissection (LCM).
We implemented this LCM technique, as presented in chapter 6, in the cell-type enriched DNA methylation analysis of bronchial, tracheal and esophageal

epithelium, and found that, in contrast to our expectations, the DNA methylation profile of the *Igf1* gene between bronchial and tracheal epithelium were mostly not distinguishable. The *Igf1* methylation pattern established in esophagus on the other hand differed widely from both bronchial as well as tracheal epithelium. The results imply, that within an organ, as it generally consists of a heterogeneous collection of different cell types, a cell-type specific methylation pattern may be masked.

By now, DNA methylation can be assessed at single-cell resolution via genome-wide bisulfite sequencing (sc-BS-seq)[25] or reduced representation approaches (scRRBS-seq)[26-28], that are interesting, although challenging, options to pursue in future research.

Igf2 & IgfBP3

As described within the previous paragraphs, IGF2 methylation profiles as well as gene expression were investigated within several studies (e.g., ref.[22,29]). In human fetal liver, higher base line *Igf2* expression levels were found for boys when compared to girls and PSE induced a reduction in male expression levels, while those in females remained unaffected[22]. In the same experiment, IGF2 DNA methylation was assessed at loci that are considered as relevant for IGF2 gene expression. Lower base line methylation was found for males at two loci, of which one was hypermethylated after PSE. For the other analyzed area, CpG-sites were found to be hypomethylated in female liver after PSE[22].

As part of our IGF network analysis in fetal and neonatal liver and lung, we also assessed *Igf2* gene expression in control and PSE offspring. Interestingly, we did not find sex-dependent base line gene expression in liver at both time points. Instead we found a PSE-induced reduction of *Igf2* gene expression in lungs of male fetuses ($p=0.04$, **Figure 1**), while females appeared to be unaffected. In neonatal lung, no sex-differences or smoke effects were detected when comparing the obtained gene expression data per organ and per time point. Interestingly, *Igf2* gene expression was higher in liver than in lung at both time points (*E17.5*: male control: $p=0.0003$, male PSE: $p=0.0001$, female control: $p=0.0009$, female PSE: $p=0.0002$; *D3*: male control: $p<0.0001$, male PSE: $p=0.0001$, female control: $p=0.0002$, female PSE: $p<0.0001$; 3rd row, **Figure 1**). Within the liver, neonatal *Igf2* gene expression increased for all subgroups when compared to that in fetuses (male control: $p<0.0001$, male PSE: $p<0.0001$, female control: $p=0.001$, female PSE: $p=0.001$); whicht is contrasted by the expression levels in lung where neonatal gene expression is lower than in fetuses in male but not in female offspring (male control: $p=0.0003$, male PSE: $p=0.0015$; females: $p=ns$; 3rd column, **Figure 1**).
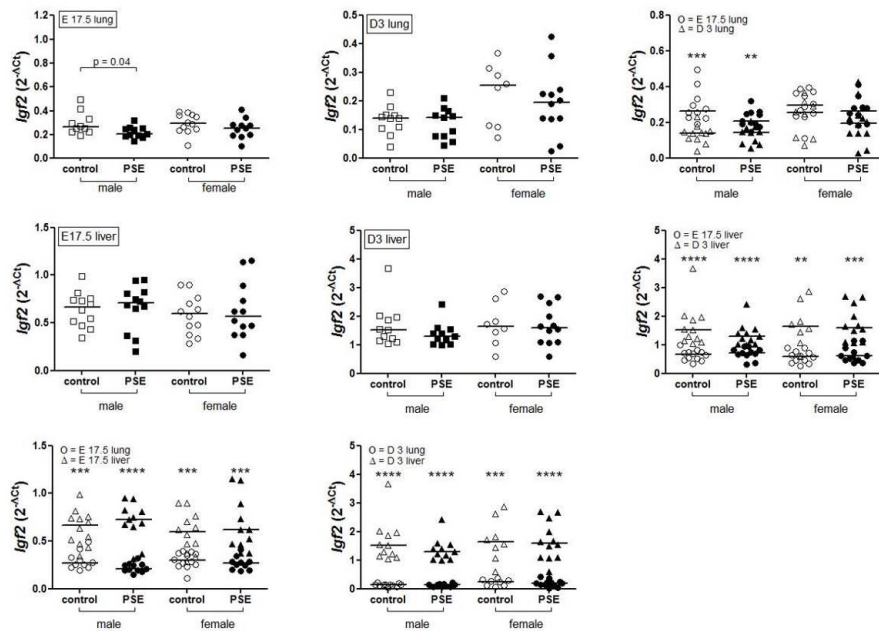
Figure 1. Sex-dependent comparison of *Igf2* mRNA levels in lung and liver of control and PSE offspring.

Data are presented per sex, organ, time point and subgroup as individual values with medians as horizontal line. If not stated otherwise, the comparison of displayed groups was not significant. *p≤0.05, **p≤0.01, ***p≤0.001, ****p<0.0001 (Man-Whitney U-test). Open symbol(s)=control group, closed symbol(s)=PSE group.

We evaluated gene expression levels for *Igfbp3*, the most abundant binding protein for both *Igf1* and *Igf2*. While *Igfbp3* is prominently evaluated in the carcinoma context[30], it hardly receives attention outside the cancer field. In an animal study in ferrets, IGFBP3 serum levels were shown to be reduced after smoke exposure[31]. We found sex-dependent base line expression in fetal liver (p=0.03, **Figure 2**). However, no smoke effect was detected and the remaining analysis in neonatal liver as well as lung at both time points did not reveal significant differences. Similar to the *Igf2* analysis, we also compared the *Igfbp3* expression levels per organ and time point. When comparing the gene expression of both organs, *Igfbp3* of lung was higher than in liver from male PSE fetuses (p=0.02; 3rd row, **Figure 2**) and female control neonates (p=0.007; 3rd row, **Figure 2**). Time point comparison revealed that neonatal *Igfbp3* gene expression in both liver and lung was higher than in fetuses and not affected by PSE (*E17.5*: male control: p=0.0001, male PSE: p<0.0001, female control: p=0.0003, female PSE: p=0.0002; *D3*: male control: 0.003, male PSE: 0.006, female control: 0.0002, female PSE: 0.0005; 3rd column, **Figure 2**).
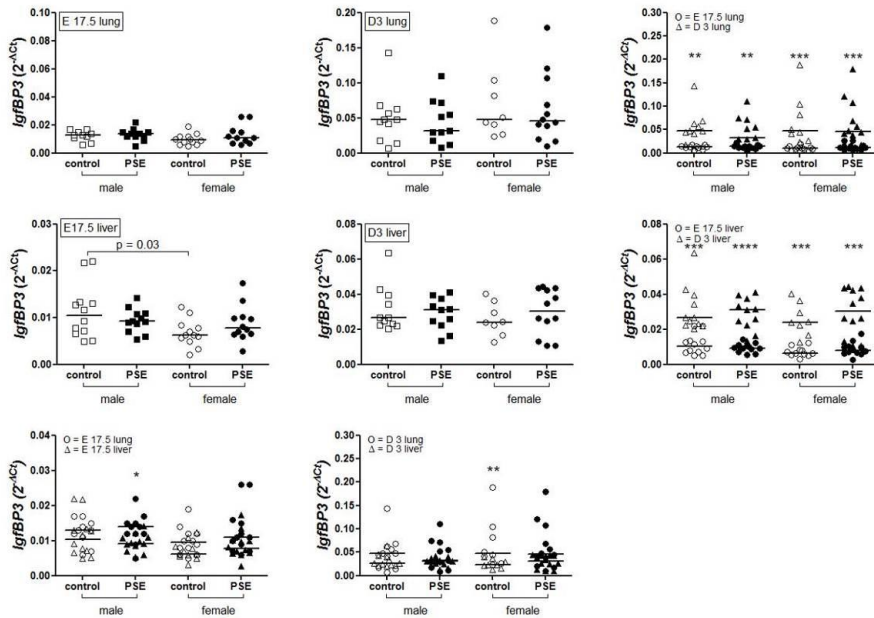
Figure 2. Sex-dependent comparison of *Igfbp3* mRNA levels in lung and liver of control and PSE offspring

Data are presented per sex, organ, time point and subgroup as individual values with medians as horizontal line. If not stated otherwise, the comparison of displayed groups was not significant. *p≤0.05, **p≤0.01, ***p≤0.001, ****p<0.0001 (Man-Whitney U-test). Open symbol(s)=control group, closed symbol(s)=PSE group.

Xenobiotic Metabolism, Cyp2a5

The metabolism of xenobiotics, nicotine in particular, was addressed in chapters 4A and 4B. We demonstrated that a PSE-induced effect on *Cyp2a5* promoter methylation is only marginal in lung of fetuses, three- and thirty-day old offspring. An additional analysis of *Cyp2a5* mRNA levels showed distinct sex-differences in lung at 30 days after birth, as PSE reduced mRNA levels in female, but not in male mice. Moreover, female baseline steady-state mRNA levels were not different from those of male mice, likely due to high inner-group variation. Sexually dimorphic *Cyp2a5* expression was reported in liver, with female mice showing higher levels than males (female/male ratio: 3.2; 32), but this information is lacking for mouse lung. However, in light of these findings, our observation that *Cyp2a5* promoter methylation is predominantly affected by PSE in fetal liver allow the speculation that the detected hypermethylation could result in reduced steady-state mRNA levels, as seen in the lung at thirty days after birth. If so, (permanently) reduced *Cyp2a5* expression levels would result in prolonged circulation of nicotine in female mice, extended nicotine-induced pharmacological effects, and ultimately minimized addictive potential of nicotine.

This is contrasted by studies demonstrating that prenatally smoke exposed children were more likely to smoke themselves[33-35] and that those showed increased addiction with more symptoms of withdrawal than children whose mothers did not smoke during pregnancy[36]. Interestingly, a "slow CYP2A6-metabolizing phenotype"was described for humans and an assessment of their smoking habits showed that slow metabolizer smokers for instance start smoking later[37], smoke fewer cigarettes per day[38,39], smoke for a shorter duration[38] and are ~2 times more likely to quit smoking[37]. These observations can be associated with several allelic variants, of which CYP2A6*9 appears to be the most common[40]. Minematsu et *al.* compared CYP2A6 genotypes in current or ex-smokers who also had COPD[41]. Except from lower life-long cigarette consumption and lower number of cigarettes per day in smokers with a genetic polymorphism of CYP2A6, Minematsu and co-workers found a protective effect of the gene mutation against pulmonary emphysema[41]. In a later study they concluded that three functional polymorphisms affected daily cigarette consumption, and the previously mentioned CYP2A6* 9 was one of them[41].

The analysis described in chapter 4 B demonstrates that the *Cyp2a5* methylation profile in liver is significantly distinct from that detected in the lung at fetal stage but also at three days after birth. This study shows as well that, when comparing both time points, the promoter methylation pattern changes significantly in the liver, and appers to be more stable in the lung. Furthermore, PSE-induced hypermethylation was most prominent in fetal liver, but the majority of PSE-induced hypermethylation was, despite a general increase in % methylation, only seen for selected CpG-sites at three days after birth. While the liver has multiple functions in mammals postnatally, the role for fetal liver is temporally limited to hematopoiesis[42]. In agreement with the report of Mäenpää et *al.* that when observing very low expression levels of P450 subfamilies 1A, 2A and 2B in fetal mouse liver[43] it is tempting to speculate on the neonatal increase of *Cyp2a5* methylation, and how the PSE-induced hypermethylation may affect gene expression and enzyme function.

Future Perspectives

Within this dissertation we set out to describe the programming effects of prenatal smoke exposure (PSE) on selected genes that were shown to play a significant role in developmental or detoxification mechanisms. Although we found some interesting PSE-induced alterations, some smoke effects were surprisingly less pronounced as expected. While we analyzed methylation profiles in gene promoters, subsequent analysis should include other putative regulatory regions, for instance enhancers[44] or equally important changes on the chromatin level (as suggested in ref. [45]). Given that several (alternative) mechanisms are involved in the regulation of transcriptional activity (e.g., histone modifications or microRNA), future studies on the

programming effect of PSE in the context of fetal and early postnatal development should also consider evaluating several of these parameters. Furthermore, time course analyses or studies that aim to compare methylation profiles in several organs or at different time points should consider the variability of cell composition per organ and that specific cell types may have a different methylation status. This also needs to be addressed when analyzing blood-derived samples.

The data presented within this dissertation are a start in studying smoke effects during pregnancy, but there are much more questions to be answered. One of these is the investigation of a possible trans- and inter-generational smoke effect. As reviewed in chapter 7, increasing evidence suggest that PSE-induced changes are trans-generationally transmitted. Recently, prenatal exposure to environmental particles was shown to increase asthma susceptibility in F1 offspring, that persisted into F2 and F3 generations, although with lesser magnitude[46]. Within this study, also aberrant DNA methylation profiles were found in dendritic cells of all generations that did not link to known allergy genes or pathways, but suggested a link to chromatin modifications.
An inter-generational consequence of prenatal smoke exposure was demonstrated in F1 and F2 generation mice that were shown with allergic asthma and bronchopulmonary dysplasia. Here, the described phenotype was linked to deregulated expression of microRNAs that are associated with, beyond others, HIF-1α-regulated immune pathways[13].

Another aspect of maternally transmitted environmental exposures during pregnancy is the synergism of several (independent) challenges, and how these may affect not just the lung but other organs in the fetus. One study addressed this question and applied maternal allergen challenge together with prenatal smoke exposure to evaluate the effect on hepatic inflammation and fibrosis in the adult F1 generation. The authors found that the allergen challenge had a greater effect on hepatic gene expression than PSE, but both exposures together enhanced the effect. Moreover, these observations were sex-dependently found for male offspring, while females appeared to be protected[47].
While the model of prenatal smoke exposure mimics active smoking, the question on possible effects of paternal smoking on the fetus remains unanswered. A Vietnamese birth cohort study linked hospitalization due to lower respiratory tract infection with paternal smoking in the presence of children[48], but other evidence for an effect on e.g., DNA methylation is missing.

Finally, it would also be interesting to clarify, if and how prenatal programming may be affected by smoking cessation or how methylation profiles in our model would differ if a prenatal smoke exposure would take place exclusively during the 1st , 2nd

or 3rd trimester. As indicated in chapter 5, variations in PSE-responses of fetuses and neonates can be linked with an additional smoking of 10 cigarettes and methylation states in human cord blood cells of key CpG-sites were associated with the number of smoked cigarettes, together with the duration of smoking during pregnancy (number of trimester)[8].

In conclusion, the data described within this dissertation indicate that maternal smoking during pregnancy affects DNA methylation profiles that can be linked to gene expression, protein levels and the offspring's body weight. As the demonstrated consequences of PSE are CpG-site specific, those identified loci may inspire for multi-organ and/or cell-type specific epigenome research.

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