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**Effects of maternal smoking
on lung function and
pulmonary signaling pathways
of the offspring:
a murine model**

Dissertation

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Abbreviations

ACN	Acetonitrile
AHR	airway hyperresponsiveness
AKT	Protein kinase B
α -SMA	alpha smooth muscle actin
ANOVA	Analysis of variance
ATI	alveolar type I epithelial cells
ATII	alveolar type II epithelial cells
bp	basepairs
BPD	bronchopulmonary dysplasia
c. section	caesarean section
CDH	congenital diaphragmic hernia
ChIP	Chromatin Immunoprecipitation
CO	carbon monoxide
COL1	Collagen 1
COPD	chronic obstructive pulmonary disease
CS	cigarette smoke
<i>D. melanogaster</i>	<i>Drosophila melanogaster</i>
Da	dalton
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxy-Nucleotide-Tri-Phosphate
DTT	Dithiothreitol
E	embryonic day
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immuno sorbent assay
ESI	Electrospray ionization
fc	Fold change
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
fwd	Forward
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GD	gestational day
GH	growth hormone
GWAS	genome-wide association study
h	hour
H1	Histone 1
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
HAT	Histone acetyl transferase
Hb	hemoglobin

Ic	Inspiratory capacity
IGF1	Insulin-like growth factor 1
IGF1R β	Insulin-like-growth factor 1 receptor beta
IGFBP3	Insulin-like growth factor-binding protein 3
IPA	Ingenuity pathway analysis
IUR	intrauterine growth retardation
K	Lysin
kDa	Kilodalton
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
m/z	Mass-to-charge
MAPK	Mitogen-activated protein kinase
miRNA	micro RNA
mRNA	messenger ribonucleic acid
MS	Mass spectrometry
NaCl	Sodium chloride
nm	nanometer
nt	nucleotides
PAGE	Polyacrylamide gel electrophoresis
PAH	Poly aromatic hydrocarbons
PAS	Periodic acid schiff
PBS	Phosphate buffered saline
PND	Postnatal day
PPAR γ	Peroxisom-proliferator-activated receptor gamma
PRAMS	Pregnancy risk assessement monitoring system
qRT-PCR	quantitative real-time PCR
rawp	raw p-value
RGZ	Resiglitazone
RNA	ribonucleic acid
rpm	rounds per minute
RT	Reverse Transcription
sc	subcutaneous
SD	standard deviation
SDS	Sodium dodecyl sulfate
SGA	small for gestational age
SNP	single nucleotide polymorphism
Stat	Signal Transducer and Activator of Transcription
TBP	TATA-box binding protein
TFA	Trifluoroacetic acid
TGF- β	transforming growth factor- β
Th	T helper
UTR	untranslated region
WHO	World Health Organisation
wt	body weight
5-mC	5-methylcytosine

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Chapter 1

Introduction

1.1 Risk factor low birth weight

Low birth weight is defined as weight at birth less than 2500 g (mean birth weight: 3500g) and very low birth weight with weight at birth <1500 g, regardless of gestational age at this time point. It is an important indicator of newborns health because of the close relationship to infant morbidity and mortality. In contrast to this, 'small for gestational age' (SGA) defines a newborn smaller in size than normal for the gestational age (<10th percentile for the gestational age). However, birth weight must be interpreted within the specific population, e.g in Indian the criteria for small birth weight is <1500 g (mean birth: 3000 g; (Murki et al., 2015)). In the USA, the number of newborns with reduced birth weight increased up to nearly 20% from 1990 to 2006 (Fig. 1.1; (Hamilton et al., 2013)). Within European countries the increase in low birth weight babies was up to 108% (Spain) from 1980 to 2010 (1.2). Low weight at birth can either occur as result of preterm birth or restricted fetal growth (UNICEF and WHO, 2004). In the US, the rate of preterm birth declined since 2012 (Martin et al., 2015). Thus, the observed higher percentage of low birth weight newborns might be caused by an increase in insufficient development and therefore indicates a poor fetal environment.

The main causes for SGA children are maternal smoking, poor maternal nutrition, young maternal age, placental dysfunction or infections. These risk factors mainly result in problems with fetal nutrition and/or oxygenation (Gluckman and Hanson, 2006). An immediate

consequence of low birth weight is neonatal mortality (49.4 death/ 1000 live birth per year; US, 2013). Furthermore, a number of long-term consequences have been described: Low birth weight has been associated with cognitive impairments, attention deficits and the need for special education (McCarton et al., 1996). Furthermore, enhanced risk for cardiovascular diseases (Rich-Edwards et al., 1997), type 2 diabetes (Harder et al., 2007) and chronic obstructive airway diseases (Anand et al., 2003) are described. Concerning obesity and diabetes, a number of epidemiological studies suggests that maternal nutrition and/or exposure to maternal glucocorticoids influences later insulin resistance or alterations in insulin secretion (Gluckman et al., 2005). A variety of observations in humans link SGA-children with an increased sensitivity for growth hormone (GH), insulin and insulin-like growth factor 1 (IGF1) and further with postnatal growth failure (Hofman et al., 1997; Gluckman and Harding, 1997). This may lead to the underlying pathophysiology of reduced fetal growth and further diseases (Fall et al., 1995; Gluckman and Harding, 1997).

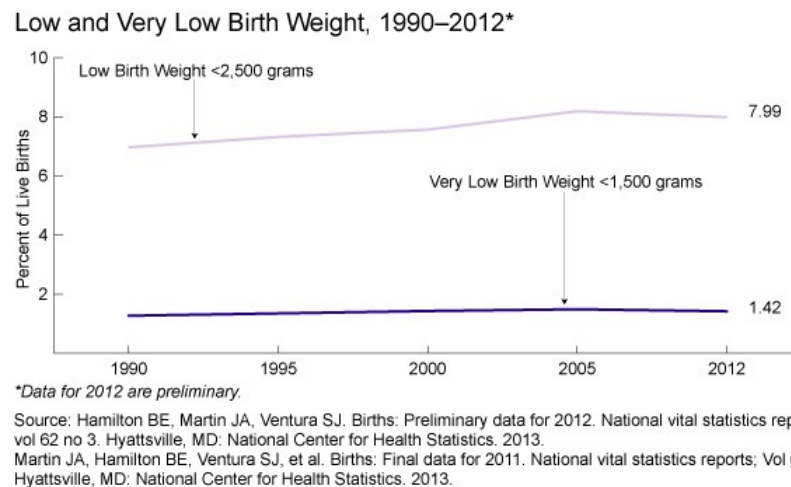


Figure 1.1: **Low and very low birth weight in the US from 1990 to 2012** OECD Health Data 2012

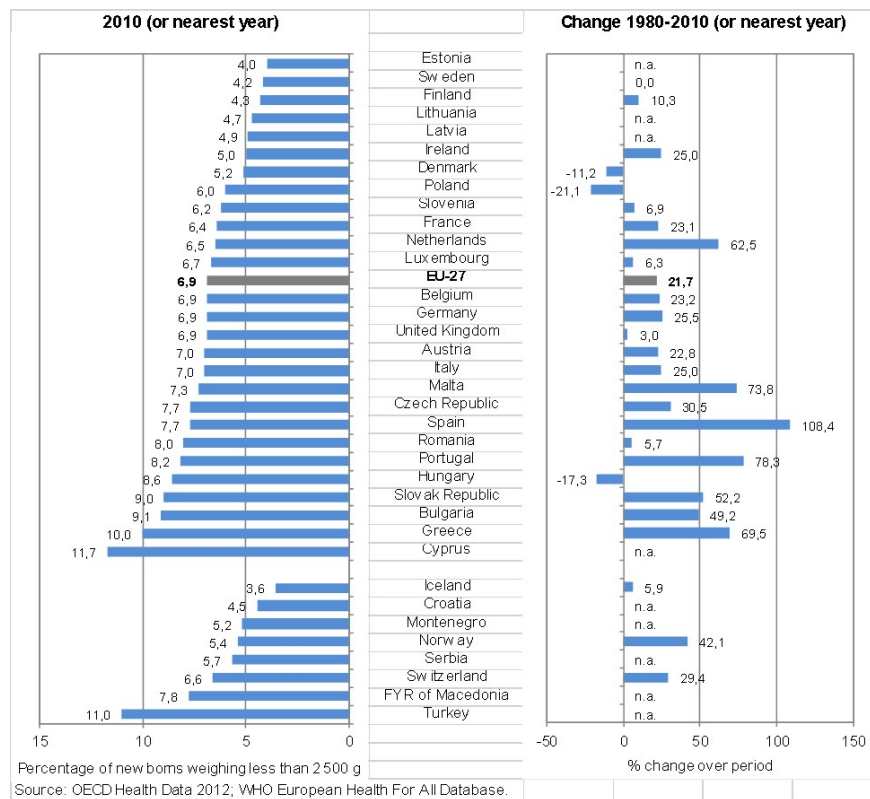


Figure 1.2: **Low birth weight infants in the year 2010 and the change from 1980 to 2010** Percentage of life birth weight low birth weight (<2500g) and very low birth weight (<1500g) OECD Health Data 2012

1.2 Low birth weight as cause for low lung function

Barker et al. (1991) reported an association between low birth weight and poor lung function. Furthermore, they linked lung function deficits with death from chronic obstructive disease in adulthood. The authors claimed, that intrauterine influences cause retarded fetal weight gain which than irrecoverably constrains the growth of the airways. Further studies supported these findings by showing that children with very low birth weight have airway obstruction (Anand et al., 2003) and reduced lung function with lower diffusion capacity (Hancox et al., 2009). Very low birth weight was further associated with reduced body weight by the age of 11 years and lower levels of expiratory flows and forced vital capacities (Kennedy et al., 2000). In addition, increased hospitalisation of very low birth weight children is mainly due

to upper and lower respiratory tract infections (Kitchen et al., 1990; McCormick et al., 1980).

The reported respiratory problems of low-birth weight infants often persist into childhood with increased respiratory morbidity, including frequent coughing and wheezing (Turner et al., 2004; McLeod et al., 1996) and may result in reduced lung function during adolescence (Lodge et al., 2014). Even in the absence of those symptoms, there may be subclinical changes which are linked to airway obstruction, hyperinflation, increased airway reactivity and exercise limitations (reviewed in Stocks, 2006). These symptoms might improve with age and somatic growth in some individuals but remain persistent in others. Morgan et al. (2005) were the first who described that the deficits in lung function in asthmatic, school-age children (16 years old) was already present at the age of six years and therefore, were not a consequence of the disease progression. In their longitudinal study the authors showed, that the children starting with poor lung function (transient early wheezing children) kept this low level until 16 years of age. Thus, a child with poor lung function at birth will stay at that level and will not improve to maximal lung function which is normally reached between 20-25 years (Tager et al., 1988). After early adulthood forced exhaled volume in 1 s (FEV₁) is stable up to 10 years and then declines throughout the rest of life. A follow-up study by Stern et al. (2007) confirmed that children born with low lung function had persistently low levels of lung function until 22 years of age. In a further approach to investigate the underlying mechanism, the association of lung function with the reaction to bronchodilators was tested. In children with low lung function at birth no improvement of lung function values after the application of the bronchodilator was found. Thus, the authors concluded that structural changes of the lung and not intrinsic airway hyperresponsiveness caused the observed airway tracking of lung function from birth onwards. They hypothesize that factors enrolled in airway development already *in utero* determine the level of airway function of the subjects. Similar results were obtained in a longitudinal study which found that persistently wheezing children in childhood continues wheezing until adulthood (26 years) and had consistently lower pulmonary function than children without wheezing (Sears et al., 2003). Furthermore, adult individuals with lung function deficits are more prone to develop chronic obstructive airway diseases (like asthma and COPD; (Weiss and Ware, 1996)). This hypothesis is strengthened by a number of longitudinal epidemiological studies, which showed that

lung function at childhood is strongly associated with bronchial hyperresponsiveness, low lung function and persistent asthma by the age of 42 years (Grol et al., 1999b,a; Vonk et al., 2004) . Svanes et al. (2010) reported that individuals with early life disadvantage (maternal asthma, maternal smoking, childhood asthma) showed permanently lower lung function and a slightly larger decline in lung function and a substantially increased COPD risk. Thus, early life environment has an essential impact on later respiratory health or disease development (Stern et al., 2007).

1.3 Long term causes of lung function deficits

As mentioned above, reduced lung function in early infancy has a major impact on the subsequent history of chronic airway disease. The term chronic obstructive airway disease describes a blockage of the airflow within the lung which is mainly caused by narrowing of the bronchi (Fig.1.3). Asthma and COPD are grouped in this term and the diseases have comparable symptoms like shortness of breath, cough, airway remodeling, inflammation and mucus hyperproduction. However, COPD is a irreversible disease of later life with a rapid decline in lung function and a high mortality rate (Weiss, 2010). In contrast to this, asthma is not a fatal disease, seen in early childhood, and is marked by consistent hyperreactivity to environmental stimuli like allergens and particles (Weiss, 2010).

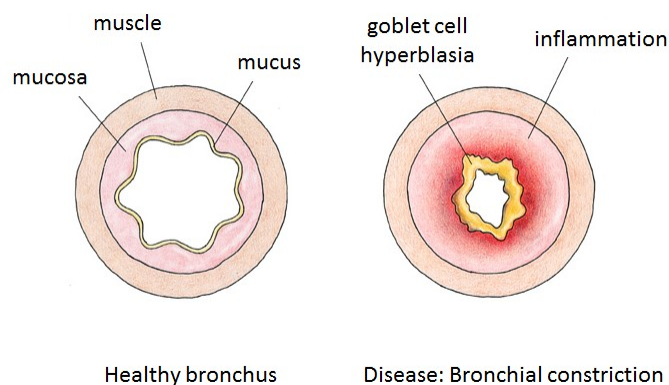


Figure 1.3: **Schematic illustration of a healthy and diseased bronchus**

As the underlying pathological features of asthma and COPD are comparable, the treat-

ment of the diseases is similar. One of the major problems associated with asthma and COPD is smooth muscle contraction called bronchospasm (Sakula, 1985). The first therapeutic approach to relax smooth muscle cells was inhalation of the bronchodilator herb *Datura stramonium*. This plant can be burned to release the acetylcholine receptor blockers hyoscyanin and scopolamine which are inhaled. A smooth muscle relaxing effect can also be achieved by intake of theophylline from tea leaves and coffee (reviewed in Boison, 2011). This is widely used within low-income countries these days as it is effective and cheap. In 1901 adrenaline was identified as strong bronchodilator and the treatment of asthma with inhalable β -adrenoceptors started (Holgate, 2013). Current bronchodilators for the treatment of asthma and COPD include long-acting β 2-agonists (salmeterol, formoterol) and ultra-long-acting bronchodilators (indacaterol) (Spyratos and Sichletidis, 2015). These drugs combine sustained smooth muscle relaxation with long duration of action (Crompton, 2006; Beeh and Beier, 2009). However, the partial efficacy of those substances led to a search for further drugs, and to the discovery of the anti-inflammatory activity of cortisone (Shampo and Kyle, 2001). Inhaled corticosteroids are of high benefit and the most important medication in asthma. Nevertheless, they are poorly effective in preventing virus-induced exacerbations (Doull et al., 1997) and they are useless in steroid-resistant asthma (Leung and Bloom, 2003; Sher et al., 1994). More fundamentally, these drugs can only cure symptoms and cannot influence the incidence of the disease as the causes are not treated.

Despite active and successful research on the pathogenesis and development of airway diseases they can still neither cured nor prevented (The Lancet, 2008). Furthermore, some patients do not respond to the medication. New insights into the initiation and the origins of the disease development are urgently needed in order to develop prevention strategies rather than merely treating the symptoms. For this approach the life course of the diseases have to be investigated by means of epidemiological studies and basic research.

1.4 Environmental and maternal influences on the development of airway diseases

Several epidemiological observations showed that the prevalence of chronic airway disease has risen over the past decades (Burr et al., 1989; Anderson et al., 1994). This observation is most pronounced in the west (Sly, 1999)(reviewed in Beasley et al., 2000). Epidemiological studies were launched to understand the underlying genetic predisposition and to identify novel candidate genes by performing linkage studies within families affected by lung function deficits and gene-wide association studies (GWAS). GWAS identified short-nucleotide polymorphism (SNPs) in several genes associated with FEV1 or FEV1/FVC (FEV1/forced vital capacity)[e.g. *TNS1* (Repapi et al., 2010); *TGFB2*, *HDAC4* (Soler Artigas et al., 2011)]. However, these findings cannot explain the increase in prevalence, as genetic alterations like SNPs are usually visible as phenotype in a population within a much longer time period than investigated in the underlying studies. Further observations in cohort studies revealed a higher prevalence in urban than in rural areas, suggesting life style-associated factors to be important for disease pathogenesis (Ernst and Cormier, 2000; Sly, 1999). Furthermore, Asher et al. (2006) observed that younger people showed a higher incidence of asthma than the older age-group indicating a higher airway hyperreactivity. After investigating the environmental conditions during early life time of the different aged groups, they found adverse environmental conditions like pollution ect. around birth for the younger people but not for the older. The fact that environmental conditions in adulthood were comparable between both groups shows that adverse environmental exposures around birth seem to have an impact. The involvement of different environmental changes might also explain the various subtypes of asthma and the different severity grades among adults and children, suggesting that there is an interplay of genetic predisposition and environmental factors. This leads to the conclusion that complex diseases, like asthma and COPD, might be a consequence of (mal)-adaptation to changing environments (Guo, 2000).

Barker et al. (1989) were one of the first to describe an association between intrauterine exposure, fetal phenotype and disease development in adulthood ("Fetal origins of adult

disease”). This hypothesis was strengthened by the observation that the offspring of severe malnourished mothers, who gave birth after the ”Dutch hunger winter” (1944), suffer proportionally more from obesity, arterial hypertension or diabetes (Heijmans et al., 2008). The rationale behind that hypothesis is, that the fetus adapts to the intrauterine environment for later postnatal conditions. However, those adaptations might also be of disadvantage when the postnatal environment has changed. Several recent cohort studies highlighted the influence of prenatal environmental changes as risk factor for an altered development leading to a number of diseases: congenital abnormalities in the offspring were linked to intrauterine herbicide and pesticide exposure (Garry et al., 1996; Yang et al., 2014), preterm birth was associated with *in utero* exposure to air pollution (SO_2) (Liu et al., 2003; Bobak, 2000) and smoking during pregnancy has been linked to fetal growth retardation (Ko et al., 2014; Horta et al., 1997). Thus, exposures during early developmental stages may also increase disease susceptibility in adulthood as it has been shown for diabetes (Ravelli et al., 1998), chronic obstructive airway disease (Svanes et al., 2010) and cardiovascular disease (Naess et al., 2013).

Already in the 90th the heritability of poor lung function was observed in twins and the authors suggested a combinatory effect of individual environmental and genetic variation as cause (Hubert et al., 1982). An example of gene environment interaction is the effect of smoking on the *GSTT1* gene loci. Low amount of cigarette smoke exposure in a *GSTT1* null genotype background was associated with low lung function in individuals, whereas effect was found in moderate and heavy smokers (He et al., 2004). This examples illustrate, that lung function and further chronic airway diseases are modulated by the interaction of individual environment and personal genetic background.

Since individual genetic variations cannot fully explain the relationship between environmental exposures in early life and disease susceptibility, it is reasonable to consider epigenetic mechanisms (reviewed in Jirtle and Skinner, 2007). Epigenetic modifications ensure the developmental plasticity of an organism, which enables the individual to react to environmental signals by changing their phenotype without alterations in the genetic base (Wolff et al., 1998; Vasicek et al., 1997). These epigenetic marks can be inherited, have long-term and short-term

effects and allow a faster adaptation to different environmental changes than genetic alterations. One of the most impressive experimental demonstration of epigenetic mechanisms came from Waterland and Jirtle (2003). They showed that a methyl-donor-rich diet (folic acid) of pregnant agouti [A(vy)] mice increased the CpG site methylation at the A(vy) locus. This change in methylation pattern of the promoter leads to alterations in ectopic agouti gene expression, which finally alters the offspring phenotype by changing the fur colour (Fig. 1.4). The authors concluded, that even small changes in nutrition during pregnancy can markedly alter the epigenetic pattern and therefore the gene expression, and lead to quite different phenotypes in adult offspring (reviewed in Dolinoy et al., 2006).

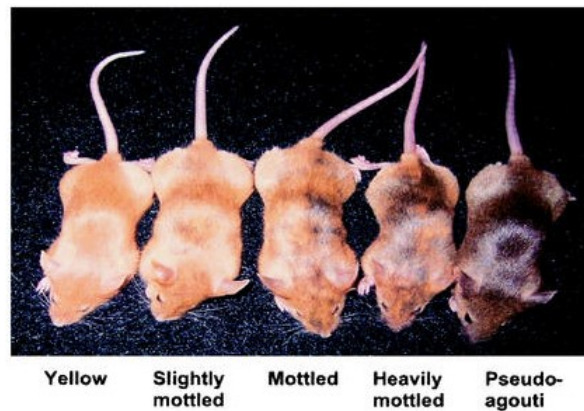


Figure 1.4: **Experimental verification of developmental plasticity** Maternal methyl supplementation changed the coat colour of Avy/a offspring. In case of hypomethylation of the Avy alleles, offspring phenotype is yellow, which refers to maximal agouti expression. Whereas Avy hypermethylation lead to silencing of the agouti gene and black fur colour is visible. (Waterland and Jirtle, 2003)

1.5 Maternal smoking as cause of impaired lung function

In European countries up to 40% of all women are smoking (WHO 2011) with even around 15-20% of these women are still smoking during pregnancy (Pregnancy Risk Assessment Monitoring System: PRAMS, USA). Cigarette smoke is composed of around 4000 compounds including nicotine, carbon monoxide (CO) and poly aromatic hydrocarbons (PAH). These

toxic substances are also transported to the fetus via the blood stream leading to a 2-fold higher nicotine concentration in the fetus than in the mother (Koren, 1995). A number of cohort studies link maternal smoking during gestation with wheezing, intrauterine growth restriction, preterm birth and poor lung function in children (Svanes et al., 2004; Gilliland et al., 2000; Kalliola et al., 2013). Furthermore, maternal smoke was also associated with structural changes in the developing lung (Elliot et al., 1998). Moreover, not only maternal smoking affected lung function in the offspring, but also grand-maternal smoking elevated the grandchild's risk of asthma indicating alterations in lung function in those as well (Li et al., 2005; Magnus et al., 2015). These changes in lung function in infants might be partly explained by alterations in gene expression of the developing fetus, which might affect lung development and maturation (Hafström et al., 2002; Sekhon et al., 1999). However, despite educational advertising and anti-tobacco campaigns the number of smoking pregnant women remain high. Concerning the observed transgenerational effects, even with successful smoking cessation programs it will take generations to restore the effect of smoking on lung function and lung disease development within the population. Therefore, it is important to develop programs to protect the growing fetal lung. For this purpose, the underlying mechanisms of how cigarette smoke affects fetal and early postnatal lung development need to be understood in detail.

First human studies investigated DNA methylation pattern to analyse the underlying causes of poor lung function after *in utero* CS-exposure. Thus, a study in children revealed different DNA methylation pattern after intrauterine CS-exposure (Breton et al., 2009). A current study by Chhabra et al. (2014) demonstrated a link between intrauterine nicotine exposure and changes in DNA methylation in fetal lung and placental tissues. Both studies confirm evidence for a modulation of epigenetic marks by cigarette smoke. Further, they provide a link between changes in DNA methylation with prenatal smoke exposure which might cause alterations in lung development and lung function deficits leading to chronic airway diseases.

1.6 Epigenetic gene regulation

Most importantly, epigenetic marks can determine the functional output of the genome without changing the DNA sequence itself. The term 'epigenetic mechanism' groups mainly two processes: 1) DNA methylation and 2) histone modifications. DNA methylation occurs at cytosine-residues converting them to 5-methylcytosine (5-mC). This alteration at CpG-sites within promoter regions can cause binding of transcriptional repressor proteins and thus conduct inactivation of the corresponding gene. Changes in DNA methylation pattern by prenatal exposures are the most studied epigenetic signal in that context and they can affect global and gene-specific DNA methylation in a tissue-specific and long-lasting manner (Breton et al., 2009).

Histones are the main structural components of chromatin and changes in the posttranslational modifications of their tails alter chromatin function and dynamics. The best studied modifications are located at the N-terminus of the histone 3 (H3) and 4 (H4) and are mainly acetylation, methylation, phosphorylation and ubiquitination 1.5. These modifications are linked to altered chromatin structure and can also affect binding of effector proteins. A tight chromatin structure causes genes to be transcriptionally inactive, while an open and accessible chromatin structure can be found in highly transcribed regions (reviewed in Krebs and Peterson, 2000). As example, acetylation or trimethylation of H3 on the lysine-residue 4 (H3K4met3) is a mark for promoter regions of active genes and leads to the recruitment of DNA polymerase II (Vermeulen et al., 2007). Gene repression can be caused by enhanced chromatin packaging which alters recruitment of repressors like the polycomb repressive complexes PRC1 and PRC2. This recruitment is directed by modification of lysine 27 in H3 (H3K27), either by mono-, di- or trimethylation (Simon and Kingston, 2009). Additional to modifications of the histone tail, nucleosome composition can alter the response to environmental changes. Mammalian H3 is grouped in two subtypes, the canonical histones (H3.1, H3.2), which are exclusively expressed during S-phase of the cell cycle and the replacement histones (H3.3, CENP-A), which are incorporated throughout the cell replication (reviewed in Bitterge and Schneider, 2014). Of note, the non-canonical H3.3 differs from the canonical H3.2 variant by only one amino acid. Nonetheless, this exchange leads to gene activation (H3.3) or repression (H3.2; (Hake and Allis, 2006)).

indicates that embryogenesis is a vulnerable stage, as DNA synthetic rate is high (reviewed in Dolinoy et al., 2007).

1.7 Lung morphogenises and molecular mechanisms

A recent study by Cuna et al. (2014) linked pulmonary DNA methylation and gene expression with lung development in humans and mice. The authors investigated lung tissue from mice with normal development and found a set of genes with inverse relationship between DNA methylation and expression. Those genes were highlighted as genes which were likely regulated by DNA methylation and were found to be mainly involved in alveolar septation process. Further, these genes were related to expression and DNA methylation data from pulmonary tissue from preterm infants with BPD, which is a human disease with disturbed alveolar septation. Thus, this publication could connect epigenetic mechanism to lung development and further to lung disorders.

In humans lung development starts around three weeks after conception with the laryngo-tracheal groove from which some days later the lung buds forms (embryonic phase; Fig. 1.6 (Groenman et al., 2005)). Further buds subdivide asymmetrically in two steps resulting in five buds each for the future lobes. In the pseudoglandular phase the tubulos-acinous gland starts to develop. The entire air-conducting bronchial tree is built up and coated with cubic epithelium as precursor cells. The respiratory ducts are formed. The canaliculi branches out of the terminal bronchioli in the canicular phase, which is characterized by alterations of the epithelium and surrounding mesenchym. Then, the acini is surrounded by capillaries which is the initiation step for later gas exchange. In the saccular phase, the sacculi are formed and coated with typ I and typ II pneumocytes. Lung development ends by forming of the alveoli out of the primary septa in infancy.

Lung development of mice is quite similar to those in humans. In the first phase (embryonic stage) the lung buds originate as an outgrowth of the foregut. Extensive branching takes place in the pseudoglandular phase and epithelial tubes are surrounded by mesenchyme. In the next step, the bronchioles are built up and capillaries arise in close contact with the cuboidal epithelium. Within the saccular phase alveolar ducts and air sacs are formed. Lung

development ends with secondary septation and alveolarization at around postnatal day 20 (alveolar phase).

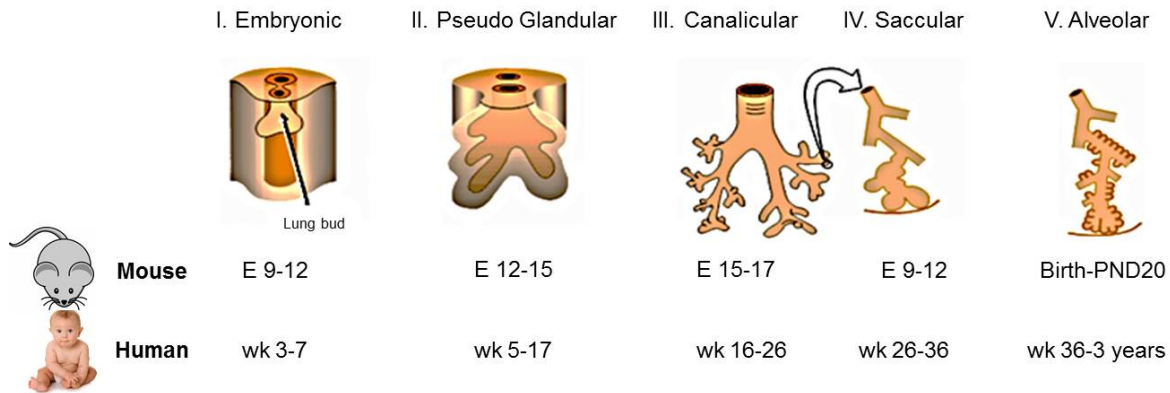


Figure 1.6: **Lung development in Human and Mouse** The five phases of lung development in mouse and human are schematically displayed with corresponding time frame after conception. E (embryonic day), PND (postnatal day), wk (week)

Thus, lungs are fully mature postnatally. This means that early environmental factors, either pre- and/ or postnatal, have a significant influence on the course of lung morphogenesis and maturation.

As lung branching and further development of alveoli, capillaries and distinct cell types is highly complex a precise regulation of gene expression is needed. First observations concerning development of airway branching were performed in the fruit fly *Drosophila melanogaster*, as this organism is quite easy to handle, has a simple physiology and a genetic organization which allows genetic manipulation (reviewed in Roeder et al., 2012). In the fly, the peptide growth factor branchless (Bnl) was found to be essential for major branching during embryogenesis (Sutherland et al., 1996) and later, the murine homologue fibroblast growth factor 10 (FGF 10) was identified (Bellusci et al., 1997). Both genes are produced by the mesoderm and regulate proliferation of the endoderm, which results in an outgrowth of the lung bud. Further proliferation and differentiation steps involves signaling of tyrosine kinase receptors mediated by: platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Additionally, growth factor mediated signaling by serin threonin receptors includes transforming growth factor β (Tgf β) and bone morphogenic protein (BMP) 4, which opposes proliferation and differentiation (reviewed in

Desai and Cardoso, 2002). For normal lung development precisely timing of the gene expression and repression is required. The accurate regulation of those developmental key genes is only possible due to the extensive combination of DNA methylation and histone modifications. Disruption of the normal epigenetic modifications in the early development can alter the gene regulation pattern and may contribute to maldevelopment of the lung (Joss-Moore et al., 2011).

1.8 Rationale

The prevalence of chronic obstructive airway diseases, is rising during the past decades and the costs for medication reach about 200 billion € per year only in Europe. Current treatments aim symptomatic relief without any curative approaches. Therefore, there is an urgent need for novel, effective therapies and prevention strategies. However, the underlying mechanism of the disease development remain entirely elusive so far. Cohort studies suggest early environmental exposures in prenatal and early postnatal development as one of the main risk factors to develop chronic lung disease later in life. In this context several risk factors like parental smoking, parental allergy or chemical exposures were highlighted without an in depth understanding of the disease-causing pathways. These observations indicate a transgenerational transmission of disease risk to contribute to the remarkable rises of chronic airway disease. The emerging field of epigenetic mechanism may provide a link between early developmental exposures and disease susceptibility and therefore, might be a starting point for prevention strategies.

Chapter 2

Aims and Objectives

Prenatal smoke exposure is a recognized risk factor for intrauterine growth restriction and impaired lung function development in children. Furthermore, it has been suggested that lung function will not improve beyond preschool years. Cohort studies suggested a high impact of environmental factors at early developmental windows (pre- and postnatally) as cause for adult diseases. This concept of early origins of disease opens the opportunities for pre- or early postnatal prevention strategies and therapies. However, to develop such approaches, the underlying molecular mechanisms need to be investigated. Therefore, this project aims to establish an intergenerational murine model for maternal smoking during pregnancy for further studying pulmonary pathways related to the intrauterine exposure. To allow the translation of findings within that model to human diseases, this murine model has to replicate the exposure levels and several phenotypic findings from human cohort studies:

- CS-exposure levels similar to those of human smokers
- unaltered parameters of a successful pregnancy as far as possible (e.g.gestational length, body weight progression of the mother, pregnancy rate, amount of abortions)
- intrauterine growth retardation of the newborns
- catch-up growth after birth
- reduced lung function of the offspring

After successful establishment of the murine smoke exposure model and confirmation of the phenotypic data, the underlying mechanism can be investigated. Therefore, molecular-biological methods will be applied to following questions:

Are there CS-related alterations in gene expression in fetal lungs?

Are there CS-related alterations pulmonary signaling pathways?

Are epigenetic mechanisms altered by intrauterine CS-exposures?

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents

Substance	Manufacturer	Country
Acetic Acid	Carl Roth	Karlsruhe (GER)
Agarose	Life Technologies	Darmstadt (GER)
Ammoniumbicarbonate (NH ₄ (CO ₃) ₂)	Carl Roth	Karlsruhe (GER)
Ammoniumpersulfate (APS)	Sigma-Aldrich	St. Louis (USA)
Bovine serum albumin (BSA)	Sigma-Aldrich	St. Louis (USA)
Bromphenolblue	Merck	Darmstadt (GER)
cOmplete mini protease inhibitor cocktail	Roche	Mannheim (GER)
Dermatophagoides pteronyssinus extract (Nr. 218234)	Greer	Lenoir, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	St. Louis (USA)
Dithiothreitol (DTT)	pjk	Kleinblittersdorf (GER)
desoxy-nucleotide (dNTP's)	NEB	Frankfurt am Main (GER)
50 bp ladder	NEB	Frankfurt am Main (GER)
Entellan	Merck	Darmstadt (GER)
Ethanol	Merck	Darmstadt (GER)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	St. Louis (USA)
Formaldehyde solution 37 %	Sigma-Aldrich	St. Louis (USA)
Formamide	Carl Roth	Karlsruhe (GER)
Formic acid	VWR	Radnor (USA)
Glycin	Applichem	Darmstadt (GER)
Hydrochloric acid (HCl), 2 M	Merck	Darmstadt (GER)
Isoflurane	cp-pharma	Burgsdorf (GER)
Ketamine	CD-pharma	Québec (CAN)
Light Cycler 480 SYBR Green I Master Mix	Roche	Mannheim (GER)

Substance	Manufacturer	Country
Magnesium carbonate (pentahydrate) (MgCO_3) ₄ Mg(OH) ₂	Carl Roth	Karlsruhe (GER)
Magnesium sulfate (heptahydrate) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Carl Roth	Karlsruhe (GER)
Magnesiumchloride (MgCl_2)	NEB	Frankfurt am Main (GER)
Mayers Haematoxylin (hemalum solution)	Merck	Darmstadt (GER)
Metacholine	Sigma-Aldrich	St. Louis (USA)
Methanol	Carl Roth	Karlsruhe (GER)
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	St. Louis (USA)
Paraffin 53-54°	Carl Roth	Karlsruhe (GER)
Pentobarbital-Na	Meridal	Hallbergmoos (GER)
periodic acid crystals	Merck	Darmstadt (GER)
Phosphate buffered saline (PBS)	Life Technologies	Darmstadt (GER)
Precision Plus Protein Standard	Bio-Rad	Hercules (USA)
Proteinase K	Roche	Mannheim (GER)
Qiazol lysis reagent	Qiagen	Hilden (GER)
RNAlater®	Life Technologies	Darmstadt (GER)
RotiBlock	Carl Roth	Karlsruhe (GER)
Rotiphorese® Gel 30	Carl Roth	Karlsruhe (GER)
Schiff's reagent	Merck	Darmstadt (GER)
Sodium bicarbonate (NaHCO_3)	Merck	Darmstadt (GER)
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich	St. Louis (USA)
Sodium chloride (NaCl)	Merck	Darmstadt (GER)
Sodium dodecyl sulfate (SDS)	GE Healthcare	Chalfont St Giles (UK)
Sulfuric acid (H_2SO_4)	Carl Roth	Karlsruhe (GER)
Trichloroacetic acid	VWR	Radnor (USA)
Tris(hydroxymethyl)- aminomethan (Tris)	Carl Roth	Karlsruhe (GER)
Triton X-100	Sigma-Aldrich	St. Louis (USA)
Tween 20	Sigma-Aldrich	St. Louis (USA)
Urea	Sigma-Aldrich	St. Louis (USA)
WesternBright ECL; WesternBright Sirius	Advansta	MenloPark (USA)
Xylazin	aniMedica	Senden-Bösensell (DE)
Xylene	Merck	Darmstadt (GER)
Xylol	Applichem	Darmstadt (GER)

3.1.2 Buffers and solutions

Name	Substance	Volume
A1	KCl	60 mM
	NaCl	15 mM
	MgCl ₂	4 mM
	HEPES (pH 7.6)	15 mM
	Triton-X-100	0.05%
	DTT	0.5 mM
	add freshly cComplete (Roche)	1 x
0.5M Tris-Cl, pH 6.8	Tris	15.41 g
	add H ₂ O bidest pH adjusted to 6.8 with HCl	250 ml
1.5M Tris-Cl, pH 8.8	Tris	45.43 g
	add H ₂ O bidest pH adjusted to 8.8 with HCl	250 ml
2x loading dye	SYBRGreenII (1:100)	50 µL
	6x loading dye	333.34 µL
	H ₂ O bidest	616.66 µL%
6x Laemmli loading dye	Tris	375 mM
	DTT	600 mM
	SDS	12%
	dissolved and pH adjusted to 6.8	
	Glycerol	60%
	Bromphenolblue	0.06%
PBND	KCl	50 mM
	Tris-HCl pH 8.3	10 mM
	MgCl ₂	2.5 mM
	gelatin	0.01 % (w/v)
	Nonidet P40	0.45% v/v
	Tween 20	0.45%
	H ₂ O bidest	to 100 mL
Periodic acid	add periodic acid cristals	0.5g
	H ₂ O bidest	100ml
	pH adjusted to 7.2 with 2M HCl	
resolving buffer (4x)	Tris	36.5 g
	SDS	0.48 g
	H ₂ O bidest	to 200 mL
	pH adjusted to 8.8	
resolving gel (1x) 7.5%	4 x resolving buffer	2 mL
	30 % acrylamide	2 mL
	H ₂ O bidest	4 mL
	10% APS	100 µL
	TEMED	12 µL

3.1.3 Buffers and solutions

Name	Substance	Volume
resolving gel (1x) 15%	4 x resolving buffer	2 mL
	30 % acrylamide	4 mL
	H ₂ O bidest	2 mL
	10% APS	100 µL
	TEMED	12 µL
stacking gel (1x)	0.5 M Tris pH 6.8	1 mL
	30 % acrylamide	480 mL
	H ₂ O bidest	2.52 mL
	10% APS	50 µL
	TEMED	12 µL
TAE buffer (pH 8.5)	Tris	242 g
	Acetic acid	57.1 ml
	EDTA, 0.5 M	100 ml
	add H ₂ O	1000 ml
TBE buffer (5x)	Tris	54.51 g
	Boric acid	27.51 g
	EDTA-Na ₂	4.65 g
	add H ₂ O	1000 ml
10x running buffer	Tris	60.6 g
	Glycin	288.2 g
	SDS	20 g
	add H ₂ O	2L
10x transfer buffer	Tris	30.3 g
	Glycin	144.1 g
	add H ₂ O	1l
10x TBS	Tris	12.1 ml
	NaCl	40 g
	add H ₂ O	500 ml
1x TBST	10x TBS	100 ml
	H ₂ O bidest	900 ml
	add Tween 20	1 ml

3.1.4 Antibodies

primary Antibody	Molecular Weight	Application	Manufacturer
α -SMA mouse mAB (A5228)	42 kDa	Western Blot	Sigma
β -ACTIN mouse mAB (3700S)	42 kDa	Western Blot	Cell Signaling
COL1 rabbit mAB (600-401-103-0.5)	130 kDa	Western Blot	Rockland
IGF1 mouse/rat mAB (ab36532)	7.5 kDa	Western Blot	Abcam
IGFR β rabbit mAB (3027S)	95 kDa	Western Blot	Cell Signaling
IGFR β P (Y1135/1136) rabbit mAB (3024S)	95 kDa	Western Blot	Cell Signaling
IgG rabbit mAB (2729P)	-	ChIP	Cell Signaling
H3K4me3 rabbit mAB (9727S)	-	ChIP	Cell Signaling
H3K27me3 rabbit mAB (9733S)	-	ChIP	Cell Signaling
secondary Antibody	Molecular Weight	Application	Manufacturer
Immun-Star goat anti-rabbit HRP-conjugate IgG	-	Western Blot	BioRad
stabilized Peroxidase conjugated goat anti-mouse IgG	-	Western Blot	ThermoScientific

3.1.5 Oligonucleotides

Primer	Acc. No	fwd (5'-3')	rev (5'-3')
<i>Igf1</i>	NM_000076.6	CTGAGCTGGTGGATGCTCT	CTCATCCACAATGCCTGTCT
<i>Igfbp3</i>	NM_008343.2	AGAACTTCTCCTCCGAGTC	GACTCAGCACATTGAGGAACT
<i>Tbp</i>	NM_013684.3	AATTGTACCGCAGCTTCAAAT	ATGATGACTGCAGCAAATCG
<i>Acta2</i>	NM_007392.3	GCTGGTGATGATGCTCCCA	GCCCATTCCAACCATTACTCC
<i>Col1a1</i>	NM_007742.3	ATCAGCTGGAGTTTCCGTGC	GGACCCATTGGACCTGAACC
<i>Kdm5c/d</i>	NM_011419.3	GGCTGACTACTTCAACATGCC	CCACTGCCAAATTCTTTGGA
<i>Igf1Prom813</i>	NM_000076.6	GCTGGTATTATTGGAATACACA	CCGTTGGCTGTATTGTTCTC

3.1.6 Commercial kits

Kit	Provider	Country
Cotinine ELISA	Calbiotech	Spring Valley (USA)
Quantitect Reverse Transcription kit	Qiagen	Hilden (GER)
SimplyChIP Plus kit	Cell Signaling Technology	Danvers (USA)

3.1.7 Consumables

Consumables	Provider	Country
Glass slides	Gerhard Menzel	Braunschweig (GER)
Histology cassettes	Thermo Fischer Scientific	Waltham (USA)
Microplate 96 well	Greiner Bio-One	Frickenhausen (GER)
qPCR plate	Biozym	Hessisch-Oldendorf (GER)
Research cigarettes 3R4F	Tobacco Research Institute	Kentucky (USA)
Shandon cytofunnels	Thermo Fischer Scientific	Waltham (USA)
Thick blot paper	BioRad	Hercules (USA)
PVDF	BioRad	Hercules (USA)

3.1.8 Devices

Equipment/ device	Name	Provider	Country
Bioanalyzer	Bioanalyzer Agilent 2100	Agilent Technologies	Santa Clara (CAL)
Centrifuges	Mikro 200	Hettich Zentrifugen	Tuttlingen (GER)
	Mikro 200R	Hettich Zentrifugen	Tuttlingen (GER)
	Rotina 420R	Hettich Zentrifugen	Tuttlingen (GER)
Cytospin centrifuge	Cytospin 4	Thermo Fischer Scientific	Waltham (USA)
Embedding Machine	Microm EC350	Zeiss	Oberkochen (GER)
Fluorescence plate reader	Sunrise®	Tecan	Männedorf (CH)
Fridge/Freezer	4°C	Liebherr	Biberrach an der Riss (GER)
	-20°C	Liebherr	Biberrach an der Riss (GER)
	-80°C	Eppendorf	Hamburg (GER)
Fume Hood	Airflow Control	Vinitex	Sint Oedenroede (NL)
Gel Imager	ChemIDoc™ Imaging System	Biorad	Hercules (USA)
Hemoxymeter	OSM3	Radiometer Copenhagen	Copenhagen (DEN)
Homogenizer	MagnaLyser	Roche	Mannheim (GER)
Homogenizer	Polytron TP2100	Thermo Fischer Scientific	Waltham (USA)
Lung function	Buxco R/C	Buxco	Troy (USA)
	FlexiVent	SCIREQ	Montreal (CAN)
Mastercycler	Mastercycler Gradient	Eppendorf	Hamburg (GER)
Microscopes	Axio Imager	Zeiss	Oberkochen (GER)
Microtom	Hyrax M55	Zeiss	Oberkochen (GER)
Nebulizer	pari Boy	Pari	Stamberg (GER)
Platform shaker	Duomax 1030	Heidolph	Schwabach (GER)
Real-time Thermocycler	Light Cycler 480	Roche	Mannheim (GER)
Scale	XS64	Mettler Toledo	Greifsee (CH)
Spectrophotometer	Nanodrop ND-1000	PeqLab Biotechnology	Erlangen (GER)
Smoking machine	modified TE-2	Teague Enterprises	Davis (USA)
Tissue processor	Microm STP420D	Thermo Fischer Scientific	Waltham (USA)
Western blot developer	ChemIDoc™ Imaging System	Biorad	Hercules (USA)
Xray developer	Curix60	Siemens Healthcare	Sankt Wolfgang (GER)

3.1.9 Software

Software	Source
Axio Vision	Zeiss, Oberkochen (GER)
GraphPad Prism v5.0	GraphPad Prism Software, San Diego (USA)
LightCycler® 480 Software v1.5	Roche, Mannheim (GER)
ImageLab™ v5.1	Biorad, Hercules (USA)
Ingenuity® Pathway Analysis	Redwood City, CA (USA)
SAS	Cary, NY (USA)
Xcalibur v2.2	Thermo Fisher Scientific, Waltham (USA)

3.2 Methods

3.2.1 Animals

General housing

Seven week old Balb/c mice were obtained from Charles River (Sulzfeld, Germany). Animals were housed in a specific-pathogen-free (SPF) facility with a 12 h day-night cycle at constant temperature (20°C) and humidity (33%). Standard rodent chow and water were provided *ad libitum*. The study was conducted under the federal guidelines for the use and care of laboratory animals und was approved by the Government of the District of Upper Bavaria.

Timed pregnancy

In the age of eight weeks, virgin females were mated with each one proven-breeder male for 24 h. As an accurate pregnancy determination was not possible 24 h after mating, all mice were initially exposed to cigarette smoke (CS). Mice were weighed every day before starting the smoke exposure protocol. When pregnancy could be excluded with certainty through body weight control the smoke exposure of the respective mice was terminated and they were killed by cervical dislocation. To allow proper implantation of the fertilized oocyte and

to avoid stress, CS exposure or air (AIR) exposure protocol was started from gestational day (GD) 2.5. CS exposure was performed on 16 consecutive days until GD 17.5 (Fig.3.1).

3.2.2 Cigarette smoke exposure protocol

Mainstream CS was used as surrogate for active smoking. Filter from research cigarettes 3R4F (Tobacco Research Institute, University of Kentucky) were removed and burned within a modified TE-2 smoking machine (Teague Enterprise; Fig.3.2). Smoke was drawn into the exposure chamber by a membrane pump. Each day, mice were exposed to ten cigarettes for 50 min, where a pump switched between 2s cigarette smoke and 4s air puff. To control the exposure the total particle concentration was measured by drawing an air sample through a quartz filter during the 3rd, 6th, 9th cigarette. The difference in the filter weight before and after the exposure was calculated in relation to the chamber volume. On average, total particle concentration was 327 ± 88 mg/m². Control animals (AIR group) were handled equally to the CS exposed animals but the cages were kept in a normal air conditioned box during the time of smoke exposure.

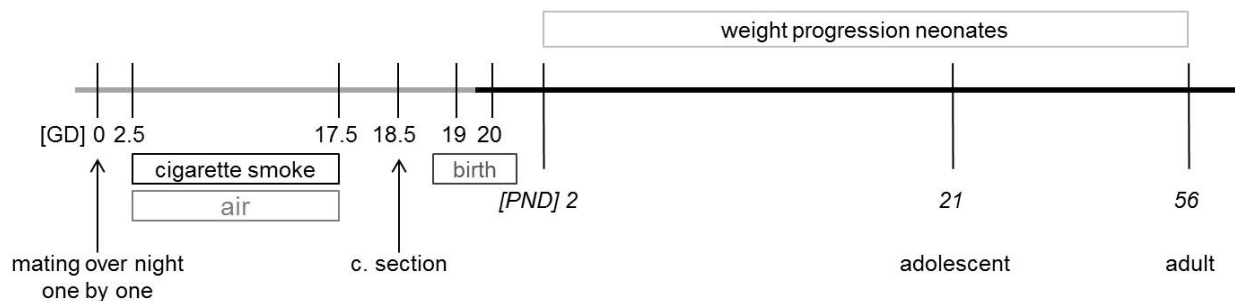


Figure 3.1: **Scheme of timed pregnancy and *in utero* cigarette smoke protocol** Eight week old female mice were exposed daily to mainstream CS starting at gestational day (GD) 2.5 for 16 consecutive days. Fetuses were either delivered by caesarean section (c.section) or naturally. At the age of postnatal day (PND)21 and PND56 mice were analysed for lung function

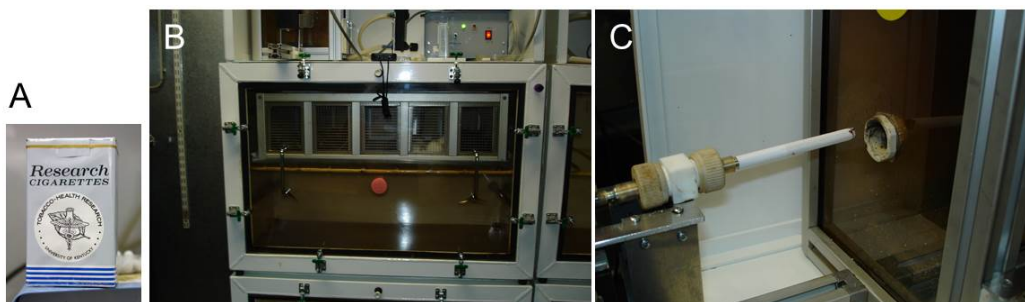


Figure 3.2: **TE-2 smoking machine** A: Research cigarettes 3R4F from the University of Kentucky B: animal chamber C: device for burning the cigarettes

3.2.3 Verification of cigarette smoke exposure

Carbon monoxide hemoglobin level measurement

To verify CS exposure, carbon monoxide hemoglobin level (CO-Hb) was analysed in non-pregnant mice. To this end, venous blood was obtained from the retrobulbar venous plexus of anaesthetized animals 30 min after the last cigarette (to allow proper air change after last cigarette) and analysed by an Hemoximeter (OSM3). As mice needed to be killed after that analysis, only non-pregnant females were used.

Cotinine analysis

For an independent confirmation of CS exposure urinary cotinine levels were determined by a Mouse Cotinine ELISA (Calbiotech). Urine was collected from non-pregnant mice four hours after CS exposure and stored in liquid nitrogen until analysis. The analysis was performed according to the manufacturer's protocol. The total cotinine amount was calculated against a standard curve. Animals did not drink during smoke exposure, to allow water intake, urine production and nicotine conversion urine was collected four hours after exposure.

3.2.4 Characterization of the offspring

For characterization of the offsprings's phenotype body and lung weights were noted at *caesarean section* (*c. section*; E18.5; fetus). Further, the lung to body weight ratios were calculated for each animal. To assess body weight development after *in utero* CS/AIR exposure

offspring were weighed daily from postnatal day 2 (PND2) to PND18. From PND18 onwards body weights were determined every week until PND59 (adulthood).

Caesarean section

Offspring were delivered by *caesarean section* at embryonic day (E) 18.5 for mRNA array analysis. For other studies, pups were delivered naturally. For *c. section*, pregnant mice were anaesthetized by subcutaneous (s.c.) administration of ketamin/xylazin in the neck fold. After opening the abdomen, the *in utero* position of viable fetuses and visible resorbed fetuses were noted. Each fetus was weighed and sacrificed by intraperitoneal (i.p.) injection of pentobarbital. The lung was removed, weighed (Mettler Toledo) and divided in two parts. The left lobe was stored in RNAlater (Life Technologies) at 4°C over night and then at -80°C until RNA isolation. The right lobes were directly frozen in liquid nitrogen. The tail was kept on ice for genotyping.

Lung function analysis

Each litter was divided in two groups of pups: the lung function of group 1 was analyzed at PND21 and lung function of group 2 was assessed at PND56. After intraperitoneal (i.p.) injection of 140 mg/kg ketamine and 7 mg/kg xylazine mice were tracheotomized and intubated (19G cannula for PND21 pups, 18G cannula for PND56 offspring). For baseline lung function analysis mice were ventilated with a tidal volume of 11 mL/kg (PND21) or 10 mL/kg (PND56) at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O on a computer controlled ventilator (FlexiVent, SCIREQ). The maximal vital capacity (MVC) maneuver was performed. For each mouse, the perturbations were performed until three acceptable measurements with a coefficient of determination (COD) 0.95 could be recorded. The mean of the measurements was then related to the body weight of each individual.

After lung function testing at baseline level, mice were transferred to a Buxco R/C system (Troy, NY). In order to assess airway hyperreactivity (AHR) in the mice metacholine (MCh) was nebulized with an in-line nebulizer in increasing concentrations (0, 12.5, 25, 37.5 mg/mL) for 20s. Resistance (R) and Compliance (C) were recorded continuously for 3 min and the

mean was calculated.

3.2.5 Molecularbiological methods

RNA Isolation

Lung tissue was disrupted in Quiazol (Qiagen) by using a bead-based homogenizator (MagNa-Lyzer, Roche). For total RNA (including small RNAs) isolation the miRNeasy Kit (Qiagen) was used according to the manufacturer's protocol.

RNA quality control

RNA quality and quantity were assessed by spectrometry (ND-100, NanoDrop Technologies) and standard agarose gel electrophoresis. For gel electrophoresis one μg of total RNA was mixed with 0.6% formamide, $0.5 \times$ TBE and SybrGreenII (1:5000) and heated for 15 min at 65°C . Afterwards RNA integrity was checked on a native 1.5% agarose gel inspecting 28S and 18S rRNA bands.

Samples used in Affymetrix Arrays were also checked by capillary electrophoresis (Bioanalyzer Agilent 2100). For this purpose, only high quality RNA samples ($\text{RIN} > 7$) were used.

3.3 Genotyping

For sex determination of the fetuses, a small piece of tail was used for DNA isolation and further PCR. To this end, the tail was digested in $200 \mu\text{L}$ PBNB buffer with $2 \mu\text{L}$ Proteinase K solution (Roche) at 56°C overnight. Next, the DNA was precipitated by adding 2.5 Vol 100% EtOH and 0.1 Vol NaOAc (3M, pH 5.2) and incubated for one hour at -20°C . After centrifugation and washing steps with 70% EtOH, the DNA pellet was air-dried at room temperature and subsequently resolved in water. For the PCR reaction 100 ng DNA, $1 \times$ Taq buffer, 2 mM MgCl_2 , 2 mM dNTPs, 0.4 pmol/ μL primer and 0.04 μL Taq polymerase (NEB) were mixed. Target sequence was amplified in a MasterCycler Gradient (Eppendorf, Hamburg) according to the protocol table 3.1. The amplified product was mixed with $2 \times$ loading dye and visualized on a 2.5% agarose gel. The expected band size for Y-linked allele *Kdm5d*

was 226 bp, whereas the size of the X-linked allele of *Kdm5d* was 255 bp.

Table 3.1: Thermal cycling condition

	°C	time (hh:mm:ss)
1	95	00:03:00
2	95	00:00:45
3	55	00:00:45
4	72	00:01:00
5	72	00:05:00
	Goto 2	35 cycles
6	10	~

3.3.1 mRNA profiling

Selection criteria for lung samples

Both androgens and estrogens can influence lung development and lung physiology (Melgert et al., 2007). To avoid this confounding factor, only lungs from fetuses were chosen, which had neighbors of identical sex (Fig.3.3). Fetuses with visible abortions within their neighborhood were also excluded from profiling.

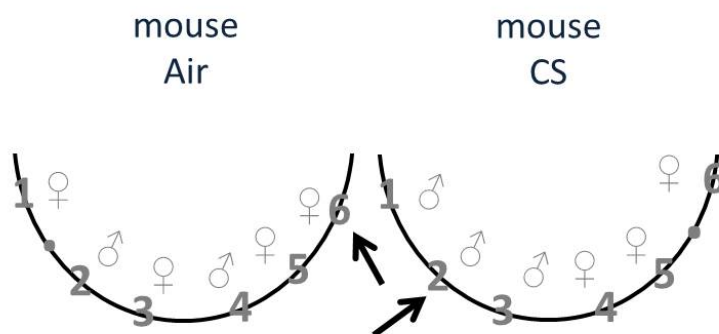


Figure 3.3: Exemplary scheme of the *in utero* position of viable pups and visible resorptions at E18.5. Numbers represent viable fetuses, while dots illustrate visible resorbed fetuses. Selected pups for mRNA profiling are labeled with arrows.

mRNA profiling

For cDNA amplification total RNA (30 ng, n = 12 per group each six males and females per group) was used in the Ovation PicoSL WTA System V in combination with the Encore Biotin Module (Nugen). Amplified cDNA was hybridized on an Affymetrix Mouse Gene ST 2.1 array plate (>28000 coding and <7000 non-coding transcripts). The plates were stained and scanned according to the Affymetrix expression protocol including minor modifications as suggested in the Encore Biotin protocol. (*This work was done by: Martin Irmeler, Institute of Experimental Genetics, Helmholtz Zentrum Munich*)

Statistical transcriptome analysis of mRNA profiling

Expression console (v.1.3.0.187, Affymetrix) was used for quality control and to obtain annotated normalized gene-level data (standard settings including median polish and sketch-quantile normalization). Redundant probe sets with identical expression values in all samples were removed from the dataset. Statistical analyses were performed by utilizing the statistical programming environment R implemented in CARMAweb (Rainer et al., 2006). The genewise testing for differential expression was done employing the (limma) t-test. (*This work was performed by: Martin Irmeler, Institute of Experimental Genetics, Helmholtz Zentrum Munich*)

mRNA correlation network

The aim of the functional analysis of a network is to highlight the biological functions and/or diseases that are most significant to the genes involved in the network. For this purpose molecular network analysis was carried out using the Ingenuity Database (Redwood City, CA, USA). MRNA expression data set was loaded into the Ingenuity Pathways Knowledge Base and a global molecular network was constructed algorithmically based on their connectivity. A Fischer's exact test was used to evaluate the significance of the probability that each biological function and/or disease assigned to that network. (*This work was performed by: Stefan Dehmel, CPC, Helmholtz Zentrum Munich*)

mRNA expression quantification by quantitative RT-PCR

One ng total RNA was converted to cDNA by using the QuantiTect Reverse Transcription Kit (Quiagen) according to the manufacturer's protocol. Specific expression patterns were analysed in 12 ng cDNA with the 1×SybrGreen II Master Mix (Roche) on a LightCycler 480 II (Roche; Tab.3.2). Data analysis comprised of baseline and threshold settings by the LightCycler 480 Software (Roche) and differential gene expression was calculated using the $\Delta\Delta$ ct-method against TATA-box binding protein (*tbp*).

Table 3.2: Thermal cycling conditions

	cycle	°C	time (hh:mm:ss)
pre-incubation	1	95	00:10:00
amplification	45	95	00:00:10
		60	00:00:15
		72	00:00:10
		78	00:00:01
melting curve	1	95	00:00:05
		60	00:01:00
cooling	1	40	00:00:10

3.3.2 Analysis of histone modifications after *in utero* CS-exposure

Histone isolation protocol

Histones were isolated from frozen lung tissue of E18.5 fetuses. To this end, 500 μ L buffer A1 was added to the tissue which was homogenised by an Ultra-Turrax (IKA) for 10 s on ice followed by a 2-fold sonification for 10 s with 10% power on ice. The sample was precipitated (30 min, 4°C, 15000 rpm) and the pellet was resuspended in 400 μ L 0.2 M H₂SO₄. The acid extraction was performed at 4°C by rocking of the samples over night. After centrifugation (10 min, 4°C, 15000 rpm), the supernatant was collected and precipitated by adding 145 μ L 100% trichloroacetic acid (TCA) followed by incubation on ice for four hours. After centrifugation (10 min, 15000 rpm, 4°C), samples were washed four times with ice-cold acetone. The pellet was air-dried at room temperature and resolved in 2x laemmli buffer, incubated at 65°C for 15 min and subsequently separated by denaturing gel electrophoresis (15% agarose gel, 110 V, 60 min). To visualize histone bands, the gel was stained with Coomassie Brilliant

Blue R-250 (0.1% Coomassie, 10% MeOH, 10% AcOH) for two hours at room temperature and destained with subsequent changes of destain-buffer (20% MeOH, 10% AcOH) for two hours. Bands corresponding to histones H3 and H4 were excised from the gel with a sterile scalpel (Fig. 3.4).

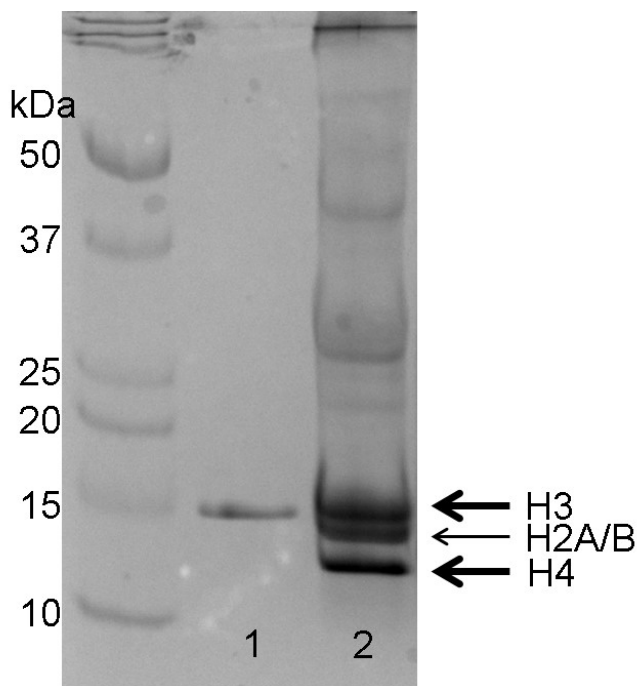


Figure 3.4: **Exemplary SDS-PAGE gel from isolated histones from whole lung tissue** Lane 1) recombinant H3 from *Escherichia coli*, Lane 2) isolated histone from whole lung tissue from E18.5 fetuses. Excised bands for mass spectrometry analysis are labeled with thick arrows.

Sample preparation for histone quantification via mass spectrometry

All chemicals used for mass spectrometry (MS) analysis were MS/HPLC grade. Gel pieces containing histones were washed twice with water and twice with 100 mM NH_4HCO_3 in order to adjust the pH value. For destaining, gel pieces were further washed three times with 50 mM NH_4HCO_3 /50 mM acetonitril (ACN) at 37°C for 10 min. Afterwards, gel pieces were successively dehydrated by subsequent incubation in 100 mM NH_4HCO_3 , 20 mM NH_4HCO_3 and three times ACN. Subsequently, samples were digested with trypsin to gain small peptides which are appropriate for MS analysis. The enzyme digests after unmodified or monomethyl-

lated lysine (K) and arginine (R) residues. The whole digestion would end up in very short (1-6 amino acid) fragments and analysis would be impractical (Fig.3.5). Therefore, lysine-residues (K) were protected from digestion by propionylation treatment that modifies unmodified and monomethylated lysines. Samples were propionylated for 45 min at 37°C (pH 7-8). To avoid over-propionylation three washing steps with 100 mM NH_4HCO_3 were carried out. Afterwards, histones were digested with trypsin (Promega) at 37°C over night. Tryptic peptides were extracted twice with 70% ACN/0.25% trifluoroacetic acid (TFA) and twice with ACN. Peptides were concentrated by vacuum centrifugation and the resulting pellet was resuspended in 0.1% TFA. Histone peptides were desalted using C18-StageTips, eluted in 80% ACN/0.25% TFA, vacuum concentrated and reconstituted in 0.1% TFA.

Tryptic peptides were injected in a HPLC system (Dionax). Peptides were separated with a gradient from 5-60% ACN in 0.1% formic acid for 40 min at 300 nL/min on a C18 column (75 μm i.d. \times 15 cm, packed in-house with Reprosil Pur C18 AQ 2.4 μm ; Doctor Maisch). The eluent was directly electrosprayed into an LTQ-Orbitrap Classic mass spectrometer (Thermo Fisher Scientific). Typical MS conditions were spray voltage, 1.5 kV; no sheath and auxiliary gas flow; heated capillary temperature, 200°C; MS1 resolution of 7500 (at 400 m/z); normalized collision-induced dissociation energy 35% (MS2) and 45% (MS3); activation $q = 0.25$; and activation time = 30 ms.

To allow quantification of modified H3 fragments a spike-in peptide (*heavy peptide*) was added to the sample. (*This work was done by Ignasi Forne, Zentrallabor fuer Proteinanalytik, Muenich*).

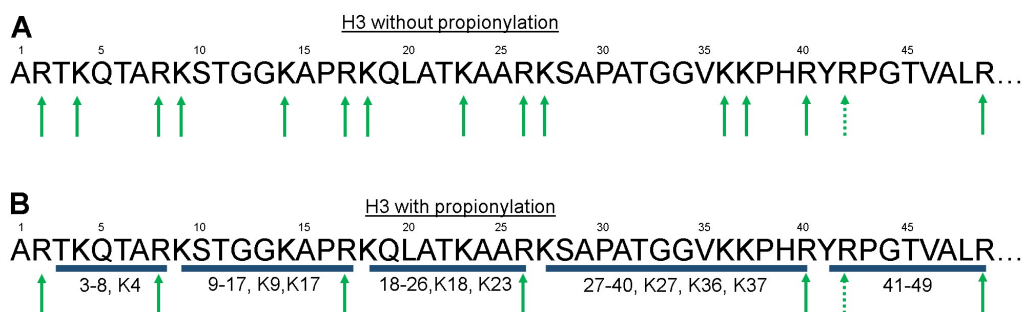


Figure 3.5: **Trypsin digestion of histone H3 with and without propionylation of lysine residues** Digestion pattern of N-terminal sequence of histone H3 without (A) and with (B) propionylation. Treatment with propionic anhydrid inhibits trypsin digestion after lysines and typical tryptic peptides of H3 are created. Arrow marks trypsin cutting site, dashed arrow marks protected arginine because of following trypsin. Numbers mark aminoacids, underline demonstrate fragments after digestion.

Protocol to analyse histone modifications obtained by mass spectrometry

The spectra of histone modifications were semi-manually quantified by the Xcalibur software package (Thermo Scientific). By using the Xcalibur Processing Setup a quantification method was build that includes the mass-to-charge ratio (m/z) and the expected retention time and allows the identification of each modification. This generated quantification method was applied to quantify the data using the Xcalibur Quan Browser. For calculation, the area below the peak from the extracted ion chromatogram was related to the corresponding spike-in (Fig.3.6). Further, the sum of all quantified modifications within one sample was calculated and the amount of each modification was depicted as percentage of all fragments.

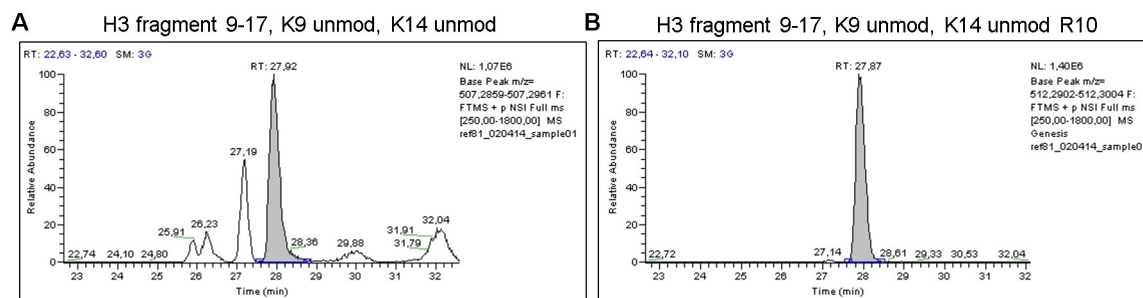


Figure 3.6: **Procedure to analyse modified H3 fragments** Chromatogram of H3 fragment 9-17, K9,K14 in unmodified form is shown in sample 1. Fragments were identified by retention time and mass-to-charge (m/z) ratio. Area below the peak in the sample (A) was related to the area below the peak in the same sample with spike-in (B)

3.3.3 Chromatin-immuno-precipitation (ChIP)

To assess the amount of repressing (H3K27met3) and activating histone modifications (H3K4met3) at the *Igf1* promoter, ChIP experiments were performed by using the SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling). In general, the manufacturer's protocol was followed with some minor modifications: as starting material 10 mg frozen lung tissue from E18.5 pups was used. Each digested chromatin sample was precleared with 20 μ L Protein-G agarose beads before starting the precipitation. The chromatin precipitation was performed with 5 μ g chromatin and an antibody concentration according to manufacturer's specifications (H3K9met3 and H3K4met3 1:100; H3K27met3 1:75) in a total volume of 500 μ L. The elution volume on the columns after DNA purification was 30 μ L. Afterwards, enrichment of the *Igf1* promoter site was validated by qRT-PCR. One reaction consisted of 1 μ L DNA with 5 μ L MasterMix (Roche), 0.6 μ L primermix (*Igf1*_Prom813) and 3.4 μ L H₂O. The standard PCR quantification program was used (Tab.3.2). For quantification the mean of each duplicated ct-value was calculated. Then, each IP was related to the input sample (%input). This value was again related to the negative control (precipitation with IgG antibody) of each sample to assess the fold enrichment.

3.4 Protein expression analysis

3.4.1 Protein quantification- according to Lowry

A piece of lung tissue was disrupted in 200 μ L 2 Laemmli buffer (including 1 PhosStop and 2M urea, freshly added) via sonification (2 x 10 s, 13% power). For protein quantification, the lysate was diluted 1:200 in H₂O and 10 μ L per sample was precipitated with 2.5 μ L 100% TCA solution for 30 min at 4°C. After centrifugation (10 min, 1000 rpm, 4°C), the pellet was resolved in 10 μ L 1 M NaOH, mixed with 31 μ L Lowry-buffer and incubated for 10 min at room temperature. This preparation was mixed with 65.5 μ L Folin-Ciocalteu-reagent (1:21 dilution) and incubated for another 30 min. The protein amount was calculated by the absorption at 750 nm and compared to a standard curve.

3.4.2 Protein detection via Western blot analysis

For protein separation a SDS-polyacrylamide gel electrophoresis was performed. 40 μ g protein of each sample in a total volume of 10 μ L was loaded to either a 15% gel (detection of IGF1, β -ACTIN) or a 7.5% gel (IGF1R β , IGF1R β P, α SMA, COL1a1). Gel electrophoresis was performed in 1 \times running buffer for 90 min at 110 V. Native PVDF membranes (BioRad) were incubated for five min in methanol before protein transfer. Proteins were transferred to the membranes for 90 min at 250 mA in 1 \times transfer buffer. After blocking of the membrane with 5% BSA (Sigma) for one hour, it was incubated with antibodies against IGF1 (1:1000 in 5% BSA), IGF1R β , IGF1R β P, α SMA, β ACTIN (all 1 : 1000 in Rotiblock), COL1a1 (1 : 2500 in Rotiblock). After over night incubation, the membrane was washed three times with TBST and incubated with HRP-coupled secondary antibody (45 min; 1 : 1000). Another washing steps were performed (3 \times with TBST) and chemical conversion of substrate was recorded with a Chemidoc XRS system. Quantification of band intensities was calculated with the Image Lab software v4.01 and normalized to β -ACTIN (loading control).

3.5 Statistical analysis

Weight gain of mothers and offspring was analysed using SAS PROCs GLM and MIXED for repeated measures analyses (SAS Institute Inc. 2011). Analysing the weight gain of animals over time requires consideration of between groups effects as well as within subject effects. Growth and group \times time interaction parameters were quantified by the natural measure milligram per day (mg/d) supplemented by 95%-confidence intervals (CI) and p-values. Corresponding synoptic graphical display of growths curves for control and exposure groups was obtained employing SAS PROC GPLOT (*performed by Hagen Scherb, Helmholtz Zentrum Munich*).

Molecular data were tested for normal distribution using D'Agostino and Pearson omnibus normality test (GraphPAD Prism). Mann-Whitney U test or t-test was applied where appropriate. Two-way ANOVA with Bonferroni posttest was conducted for AHR.

Chapter 4

Results

4.1 Establishing a model of prenatal cigarette smoke exposure

First, exposure to mainstream cigarette smoke was evaluated (blood carboxy-hemoglobin (CO-Hb) and urine cotinine value). Afterwards, as part of our evaluation of a successful pregnancy, parameters like maternal body weight development, litter size and pregnancy rate were assessed.

4.1.1 Characterization of cigarette smoke exposure

To confirm the exposure of the female mice, blood CO-Hb and urinary cotinine (metabolite of nicotine) levels were explored. As depicted in figure 4.1 cotinine as well as CO-Hb level were significantly increased in CS-exposed mice compared to control animals. The mean CO-Hb value (6.3%) is comparable to human smokers who smoke 11 to 20 cigarettes per day (Castleden and Cole, 1975). The absolute cotinine values within our model are comparable to <10 cigarettes/ day in humans (Matsumoto et al., 2013).

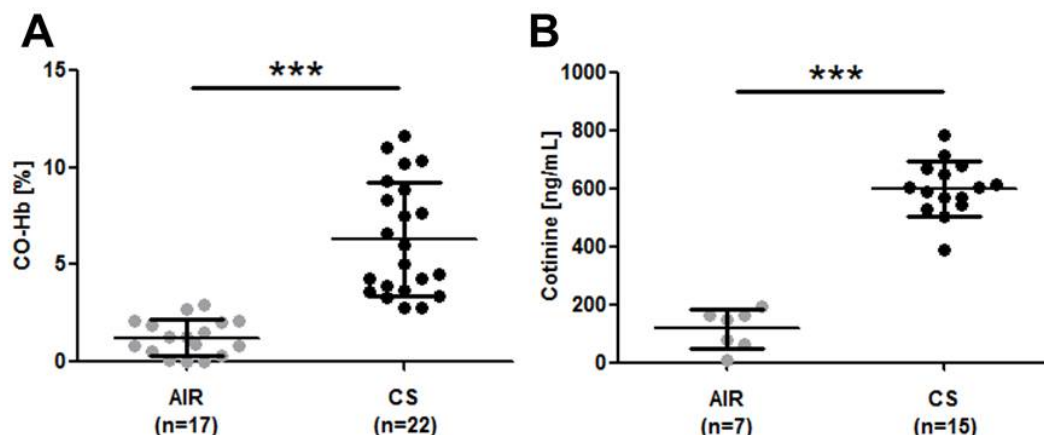


Figure 4.1: **Urine cotinine and carbon monoxide hemoglobin level of mainstream CS-exposed mice.** Analysis of CO-Hb (A) and cotinine (B) levels of non-pregnant mice. Blood withdrawal for CO-Hb measurement was done 30 min after AIR (grey dots)- or CS (black dots)-exposure. Urine sampling was conducted four hours after CS-exposure. Data of three independent measurements are shown and statistical analysis was done using unpaired t-test with $***p \leq 0.001$. Bars indicate mean \pm SD

4.1.2 Pregnancy characteristics

To discriminate between pregnant and non-pregnant mice and to further assess, if CS affects maternal weight gain in pregnancy, body weights were recorded daily (Fig.4.2). A significant difference in body weight between pregnant and non-pregnant animals became apparent on day 12.5 (4.2). In non-pregnant mice CS-exposure resulted in a significant decreased body weight gain by 80 mg/d compared to control animals (95% CI: 75-84, $p < 0.0001$; Fig.4.2/A). Furthermore, regression analysis of the body weight gain over time and exposure revealed that weight gain in pregnant CS-exposed females was significantly decreased by 157 mg/d compared to pregnant AIR-exposed females ($p < 0.0001$; 4.2/B).

We wanted to avoid secondary effects due to a compromised pregnancy. Therefore, several general pregnancy parameters like pregnancy duration, litter size and visible resorbed fetuses were investigated. As depicted in table 4.1 no difference was observed for pregnancy rates ($12.4 \pm 7.3\%$ in AIR vs $12.6 \pm 8.6\%$ in CS; mean \pm SD) or pregnancy duration (19.8 ± 0.6 d in AIR vs. 20.1 ± 0.7 d in CS) for both groups. Furthermore, the number of viable pups per litter of AIR-exposed mothers (5.9 ± 1.7) was comparable with those of CS-exposed animals

(5.4 ± 1.9). No statistically significant difference was seen for the number of visible resorptions per litter (0.6 ± 0.8 vs 1.0 ± 0.8). Likewise, the percentage of male offspring was similar in CS-exposed and control mice.

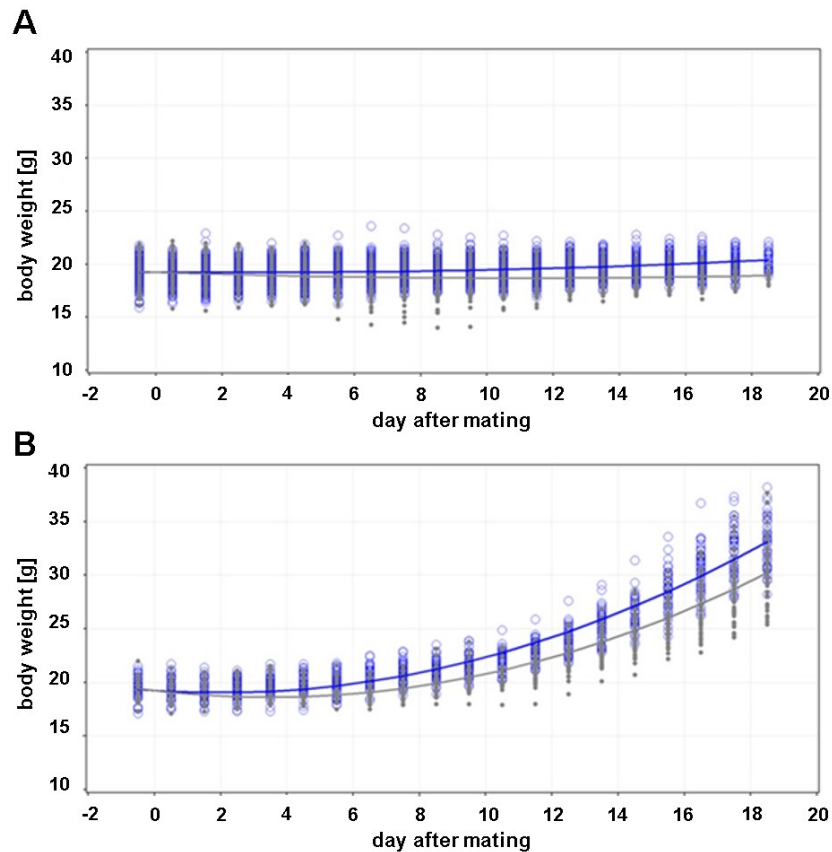


Figure 4.2: **Body weight development of non-pregnant (A) and pregnant (B) female Balb/c mice after AIR- or CS-exposure** Body weight development of CS-exposed females is significantly decreased than of AIR-exposed mice ($p < 0.0001$) in the non-pregnant and pregnant group. Mice were weighed daily before AIR (blue)- or CS (grey)-exposure. The daily exposure started at day 2.5 and ended at day 17.5 after mating. Data of $n \geq 48$ females (≥ 20 batches) are depicted and statistical analysis was done using regression analysis with SPSS and were not adjusted for litter size (*performed by H. Scherb*)

Table 4.1: **Pregnancy Characteristics**

Parameter	AIR	CS	p-value
Pregnancy rates (c-section & natural birth) [%] mean \pm SD (n, mating batches)	12.4 \pm 7.3 (20)	12.6 \pm 8.6 (23)	0.95
Pregnancy duration (natural birth) [d] mean \pm SD (n, dams)	19.8 \pm 0.6 (28)	20.1 \pm 0.7 (47)	0.11
Viable pups per litter (c-section & natural birth) mean \pm SD (median; n, litter)	5.9 \pm 1.7 (6.0; 48)	5.4 \pm 1.9 (6.0; 67)	0.15
Visible resorptions per litter (c-section) mean \pm SD (n, litter)	0.6 \pm 0.8 (20)	1.0 \pm 0.8 (20)	0.14
Male offspring (c-section & natural birth) [%] mean \pm SD (n, litter)	46.2 \pm 17.5 (38)	43.6 \pm 27.4 (52)	0.47
Maternal weight at E18.5 [g] mean \pm SD (n, dams)	33.3 \pm 2.5 (41)	30.5 \pm 2.8 (55)	<0.0001
Maternal weight at E18.5 (LS > 6) [g] mean \pm SD (n, dams)	35.2 \pm 1.7 (17)	32.3 \pm 2.4 (21)	<0.0001
Maternal weight at E18.5 (LS \leq 6) [g] mean \pm SD (n, dams)	32.0 \pm 2.1 (24)	29.4 \pm 2.4 (34)	<0.0001

4.2 Phenotypic analysis of the offspring

The offspring of AIR- or CS-exposed Balb/c mice were analysed at three distinct time points of development: embryonic day [E]18.5 (fetal stage), postnatal day [PND]21 (end of breast feeding, adolescence) and PND56 (adulthood)[Fig.3.1]. Phenotypic assessment included lung and body weight analyses and lung function analyses. Furthermore, remodeling of the lung was assessed by expression and protein analysis of α smooth muscle actin (α SMA) and Collagen1 (Col1).

4.2.1 Fetal phenotype (E18.5) after intrauterine CS-exposure

Usually, the litter size of mice varies between one to ten pups. Litter size distribution within our model is shown in Figure 4.3. The allocation of the different litter sizes is comparable between AIR- and CS-exposed dams and the calculated median is six for both groups. Litter size might have an effect on nutrition status of the mother and nutrient supply of the pups. To achieve an uniform distribution of the groups, phenotypic data of the fetuses (E18.5) were analysed according to litter size larger than six and smaller or equal to six. Figure 4.4 shows fetal phenotypic data of all animals irrespective of litter size. Female CS-exposed fetuses had highly significant lower body (0.89 ± 0.11 g; mean \pm SD) and lung weights (27.81 ± 6.62 mg) compared to control animals (body weight 1.00 ± 0.16 g and lung weight 33.38 ± 7.06 mg; $p < 0.001$). On the other hand, body and lung weight of male offspring were not significantly affected by intrauterine CS-exposure. After calculation of the lung-to-body weight ratio only the values for CS-exposed females were significantly decreased in comparison to the values of AIR-exposed mice ($31.10 \pm 5.64 \frac{mg}{g}$ in CS vs. $33.45 \pm 4.97 \frac{mg}{g}$ in AIR) indicating a relatively higher deficit in lung growth.

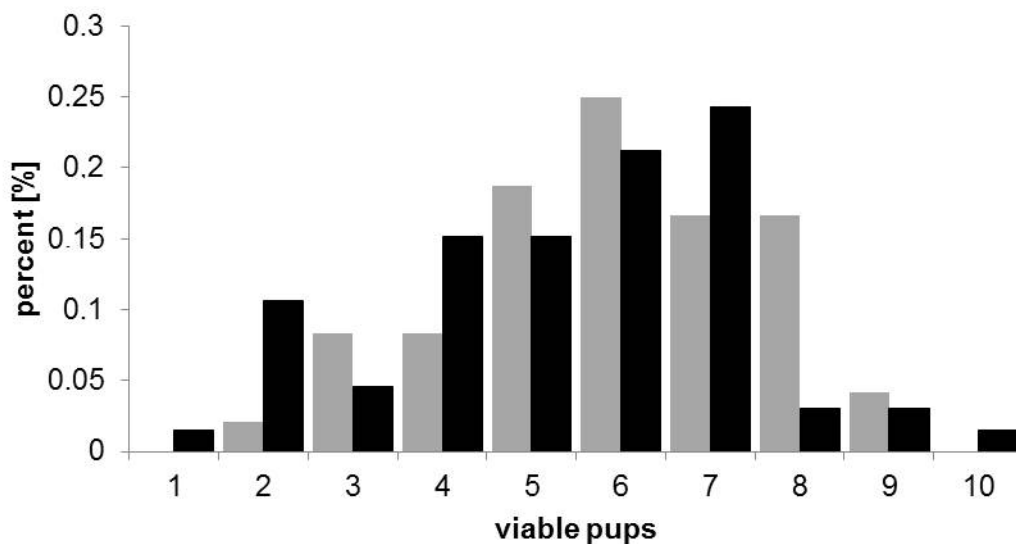


Figure 4.3: **Histogram of litter size distribution of viable pups** Litter size in percent of AIR-(gray) and CS-(black) exposed dams with $n \geq 48$ per exposure (≥ 20 batches)

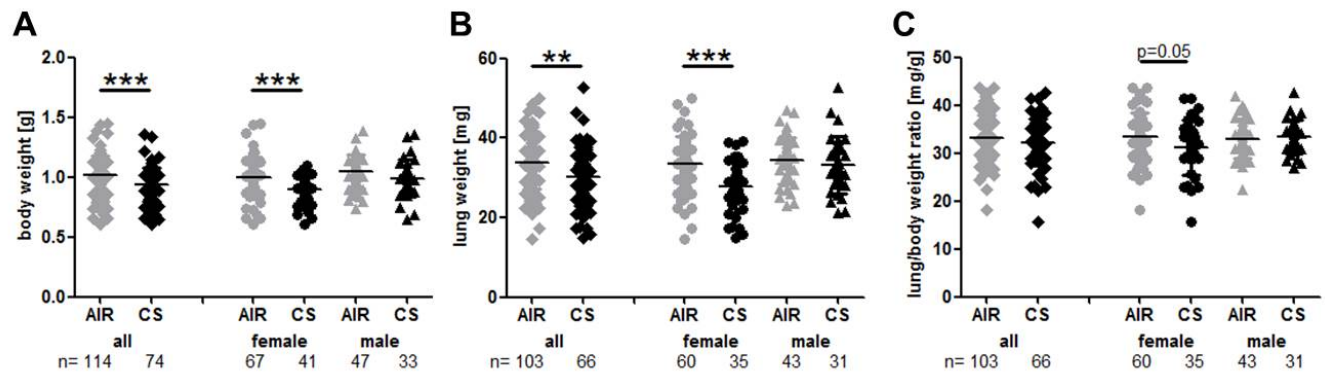


Figure 4.4: **Phenotype of *in utero* cigarette smoke exposed fetuses at embryonic day 18.5 (all litter sizes included)** Body weight (A), lung weight (B) and lung-to-body weight ratio (C) are displayed according to sex and exposure. Fetuses were delivered by *caesarean section* on E18.5 24 h after the last exposure to AIR (gray) or CS (black). Circles (\circ), triangles (\triangle) or diamonds (\diamond) indicate females, males or both, respectively. Data of $n \geq 31$ (≥ 6 batches) are depicted and statistical analysis was done using Mann-Whitney-U test with ** $p < 0.01$, *** $p < 0.001$

When restricting the analysis to litter size > 6 , the body weight of male pups was also significantly decreased by intrauterine CS-exposure [Fig.4.5] (1.03 ± 0.13 g in AIR vs. 0.93 ± 0.08 g in CS; $p < 0.05$; mean \pm SD). However, this was more pronounced in females (0.98 ± 0.16 g in AIR vs. 0.88 ± 0.11 g in CS; $p < 0.01$). Looking at litter sizes below or equal to six only the female body weight was significantly reduced in CS- compared to AIR-exposed animals (Fig.4.6) (0.92 g \pm 0.12 vs. 1.07 g \pm 0.17 ; CS and AIR, respectively). Lung weight and lung to body weight ratio were unaffected.

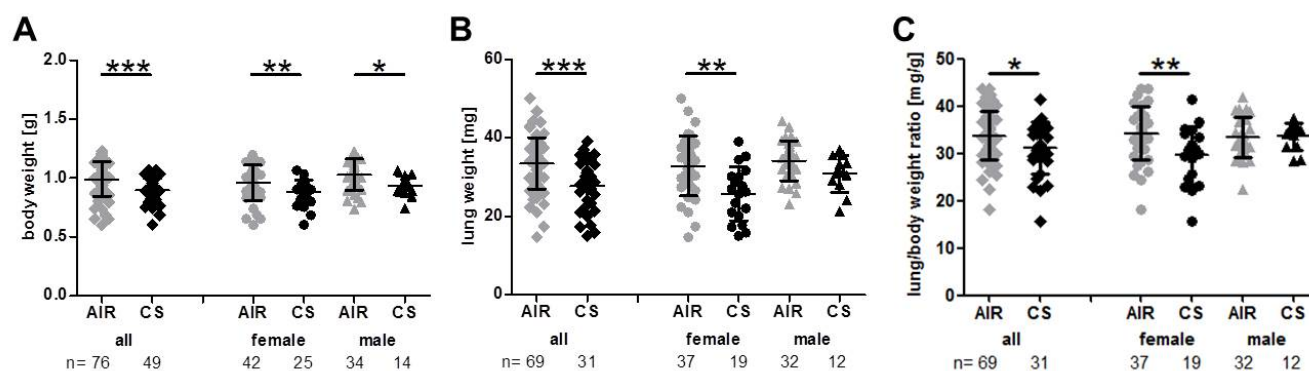


Figure 4.5: **Phenotype of intrauterine cigarette smoke exposed fetuses with litter size above six** Body weight (A), lung weight (B) and lung-to-body weight ratio (C) are shown according to sex and exposure (AIR [gray] or CS [black]). Circles (\circ), triangles (\triangle) or diamonds (\diamond) indicate females, males or both, respectively. Data of $n \geq 12$ (≥ 6 batches) are depicted. Statistical analysis was done using Mann-Whitney-U test with * $p < 0.05$, ** $p < 0.01$

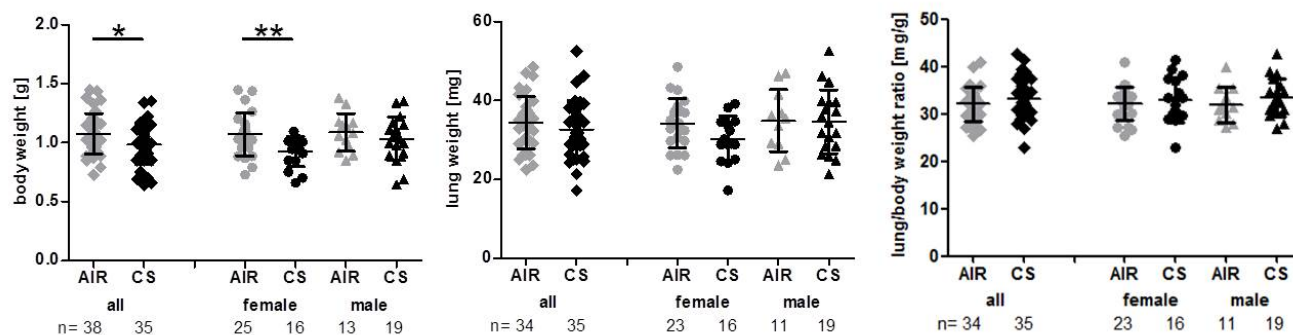


Figure 4.6: **Phenotype of intrauterine cigarette smoke exposed fetuses with litter size below or equal six** Body weight (A), lung weight (B) and lung-to-body weight ratio (C) are shown according to sex and exposure (AIR [gray] or CS [black]) with a litter size ≤ 6 . Circles (\circ), triangles (\triangle) or diamonds (\diamond) indicate females, males or both, respectively. Data of $n \geq 12$ (≥ 6 batches) are depicted. Statistical analysis was done using Mann-Whitney-U test with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

4.2.2 Postnatal body weight gain after *in utero* CS-exposure

Body weight gain of the pups after intrauterine CS-exposure was followed from PND2 to PND59. The day of birth was defined as day 0 (Fig.4.7). Analysing of the regression curve revealed that CS-exposed offspring were significantly lighter than control mice over the time in both sexes ($p < 0.001$; adjusted for time, litter size and sex, *analysis performed by H. Scherb*). However, amount of included animals in this analysis highly differ between early postnatal days (e.g. PND5: $n \geq 58$, ≥ 12 batches) and later postnatal days (e.g. PND56 $n \geq 5$, ≥ 3 batches). Furthermore, the end of weaning can be seen in a delayed body weight gain between PND15 to 25. Of note, the interaction of litter size with smoke exposure over time was not significant and hence was not included in the model.

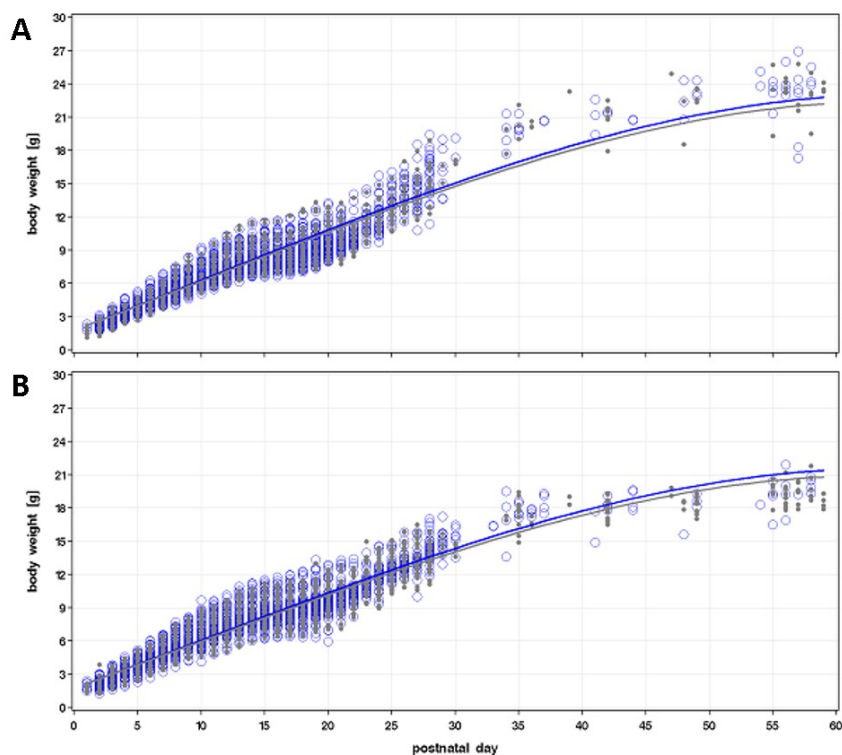


Figure 4.7: **Body weight development after intrauterine CS-exposure until adulthood** Body weight is shown according to sex (male (A) and female (B)) and exposure (AIR [blue]; CS [gray]). In both sexes pups showed significantly reduced body weight gain after intrauterine CS-exposure compared to control animals ($p < 0.001$). Assessment of body weight was done daily beginning at day 2 after birth until day 58. Data of $n \geq 5$ (≥ 3 batches) are depicted and statistical analysis was done using Analysis of Variance in SAS (*performed by H.Scherb*)

4.2.3 Lung function analysis of CS-exposed pups

Lung function parameter in adolescent offspring (PND21)

In order to investigate the effects of intrauterine CS-exposure on lung function, the inspiratory capacity per body weight [Ic/wt] and airway hyperreactivity [AHR] were assessed. As depicted in Figure 4.8 the inspiratory capacity was significantly decreased in pups after *in utero* CS-exposure in both sexes (50.64 ± 2.71 mL/kg vs. 42.55 ± 3.44 mL/kg in females and 47.97 ± 3.27 mL/kg vs. 43.92 ± 2.49 mL/kg in males; AIR vs. CS, respectively; mean \pm SD). However, a significant increase in AHR after stimulation with metacholine (at

25 and 37.5 mg/mL) was only present in CS-exposed females compared to control mice (25 mg/mL: $2.81 \pm 0.12 \text{ cm H}_2\text{O}^*\text{s}^*\text{mL}^{-1}$ in AIR, $3.42 \pm 0.42 \text{ cm H}_2\text{O}^*\text{s}^*\text{mL}^{-1}$ in CS, $p < 0.01$; 37.5 mg/mL: $3.13 \pm 0.21 \text{ cm H}_2\text{O}^*\text{s}^*\text{mL}^{-1}$ in AIR, $3.71 \pm 0.49 \text{ cm H}_2\text{O}^*\text{s}^*\text{mL}^{-1}$ in CS, $p < 0.01$). Nevertheless, AHR in males showed the same trend but did not reach statistical significance due to high standard deviation.

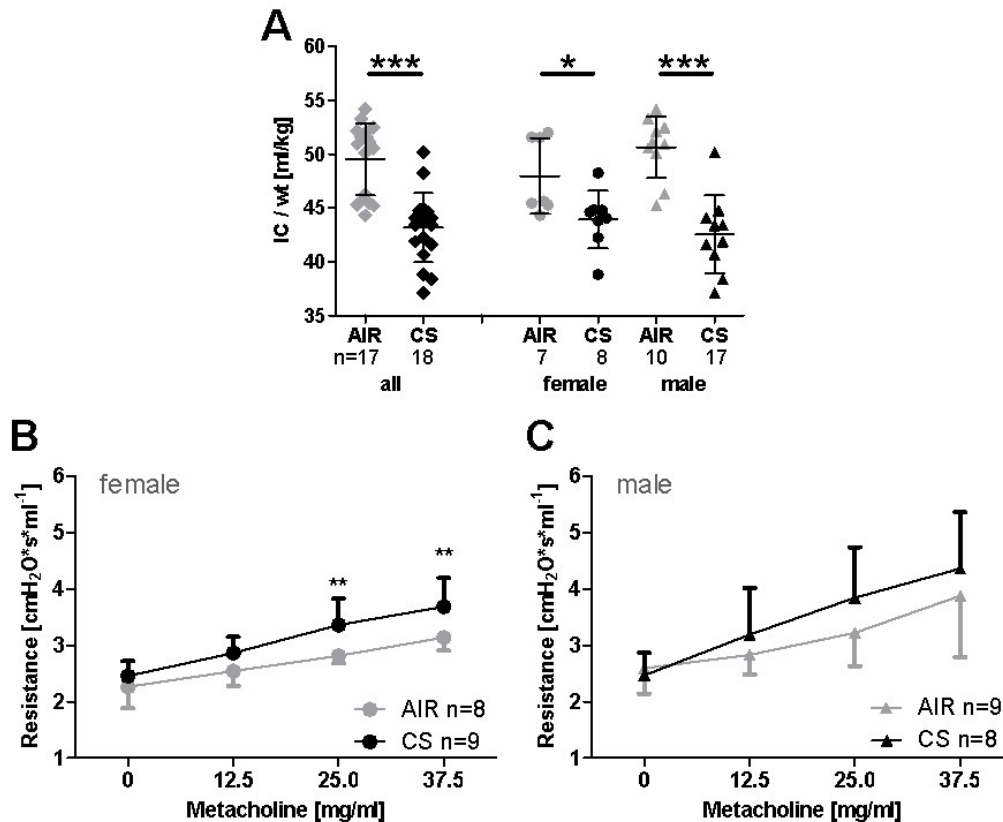


Figure 4.8: Lung function analyses of adolescent pups after intrauterine exposure to CS. Inspiratory capacity per body weight (Ic/wt; A) and airway hyperresponsiveness (AHR; B-C) after metacholine challenge at PND21 after *in utero* exposure to AIR (grey) or CS (black). Ic was assessed by flexiVent system, while AHR was measured using an invasive Buxco R/C. Circles (\circ), triangles (\triangle) or diamonds (\diamond) indicate females, males or both, respectively. Data of $n \geq 7$ (≥ 3 batches) are shown and statistical analysis was done using Mann-Whitney-U test with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ for Ic/wt and two-way ANOVA with Bonferroni posttest for AHR.

Lung function in adult mice (PND56)

To assess if lung function deficits track into adulthood, lung function analyses were further performed at the age of 8 weeks. At this age, all investigated lung function parameters had returned to normal. Mice showed no effect of intrauterine CS-exposure neither in Ic/wt nor in AHR after metacholine challenge (Fig.4.9).

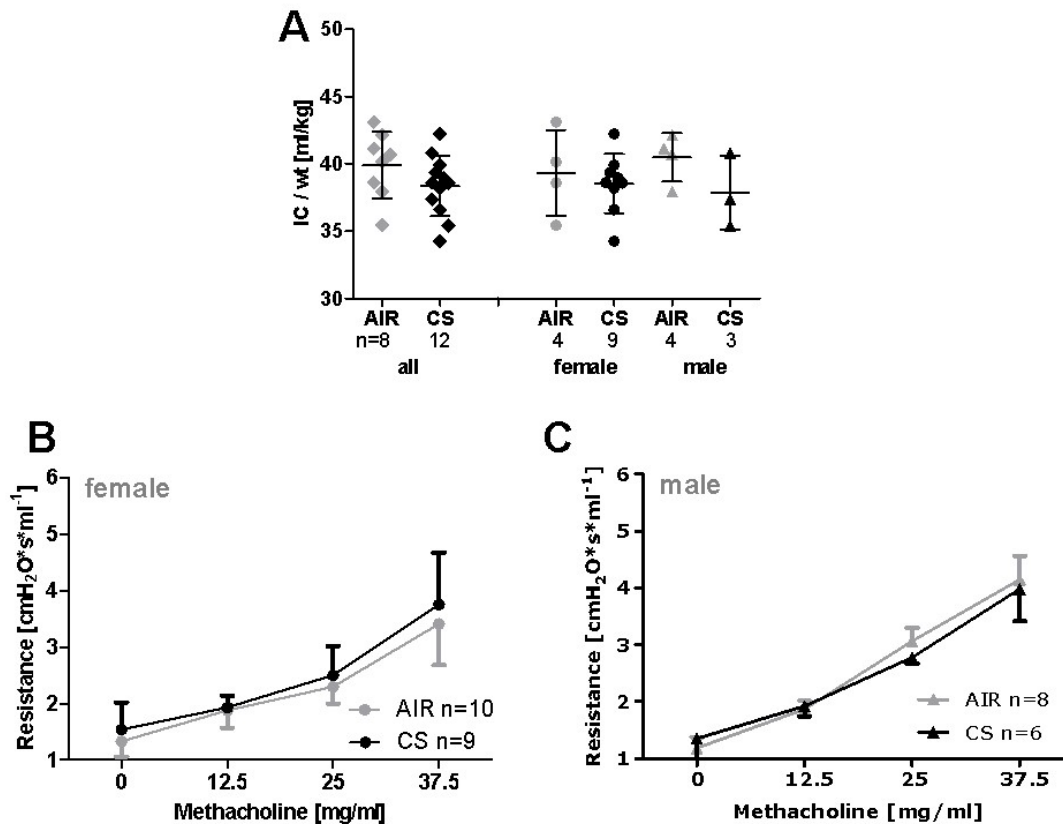


Figure 4.9: **Lung function analyses of the offspring after at PND56 after *in utero* CS-exposure** Inspiratory capacity per body weight (Ic/wt; A) and airway hyperresponsiveness (AHR; B-C) after metacholine challenge at PND56 after *in utero* exposure to AIR (grey) or CS (black). Ic was assessed by flexiVent system while AHR was measured using an invasive Buxco R/C. Circles (\circ), triangles (\triangle) or diamonds (\diamond) indicate females, males or both. Data of $n \geq 3$ (≥ 1 batches [Ic/wt]; ≥ 3 batches [AHR]) are shown and statistical analysis were done using Mann-Whitney-U test with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ for Ic/wt and two-way ANOVA with Bonferroni posttest for AHR

4.2.4 Airway remodeling after *in utero* CS-exposure

To investigate if airway remodeling occurred after intrauterine CS-exposure, mRNA expression as well as protein levels of α -smooth muscle actin (α -SMA) and collagen 1 (Col1) were analysed. Only small changes were observed in *Col1* expression in whole lung tissue during the different developmental points (Fig.4.10 [A-C]). At PND21 *Col1* was significantly up-regulated without stratifying for sex (fc = 1.4, p < 0.05). In contrast, mRNA was down-regulated (fc = -1.8) at PND56 in male pups with low significance (p < 0.05). Nevertheless, the expression profile is in line with COL1 protein level, where no effect of CS-exposure was detected in all investigated time points in females. COL1 could not be detected in males.

exposure at the investigated growth stages (Fig.4.11 A-C). However, Western blot analyses revealed an CS-effect in male pups at all observed developmental stages (Fig.4.11 D-F). Protein level in males of α -SMA was significantly elevated with a $fc = 1.4$ ($p < 0.01$, E18.5) and 1.9 ($p < 0.01$, PND21) while a trend for increasing α -SMA can be seen at PND56 ($fc = 2.9$, $p = 0.09$) albeit without significance due to high standard deviation.

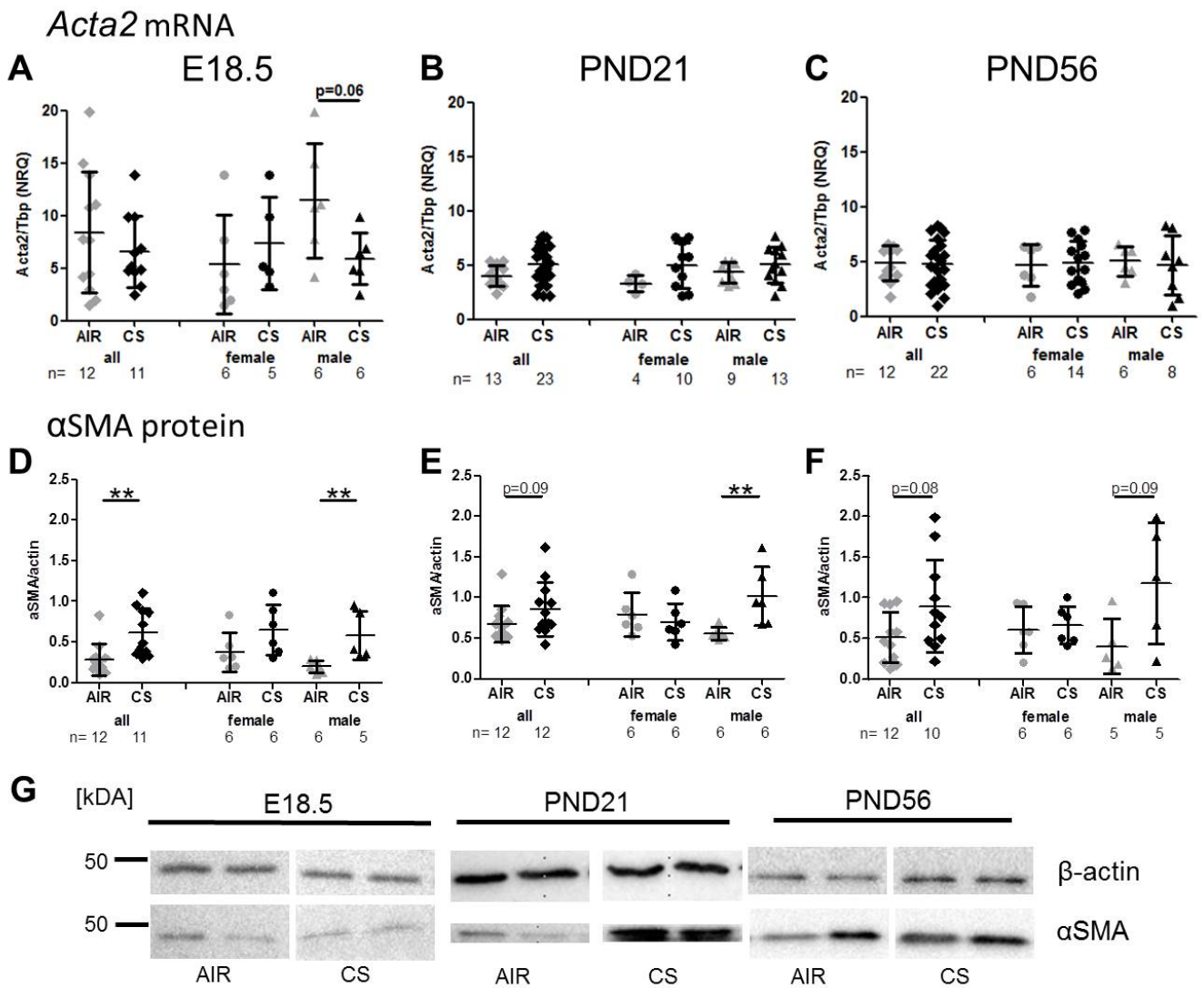


Figure 4.11: **Expression and protein level of α -smooth muscle actin after *in utero* CS-exposure** Expression (A-C) and protein level (D-F) of α -SMA in whole lung tissue on different developmental stages. Expression level were determined by qRT-PCR with *Tbp* as reference gene and protein level were calculated by Western blot analyses with β -ACTIN as reference. (G) representative Western blot. Pups were exposed intrauterine to AIR (black) or CS (gray) and circles (\circ),triangles (Δ) or diamonds(\diamond) indicate females, males or both. Data of $n \geq 4$ (≥ 3 batches) are shown and statistical analyses were done using Mann-Whitney-U test with $**p < 0.01$

4.3 Alterations of pulmonary gene expression profile in CS-exposed fetuses

After having established a murine model of prenatal cigarette smoke exposure and confirmation that the phenotype is comparable to observations made in children, molecular analysis were performed.

4.3.1 Analysis of mRNA expression profile (*performed by M. Irmeler and S. Dehmel*)

Within the mRNA array data, three samples (AIR-treatment, one male, two females) were identified as outliers and excluded from further analysis. Thus, expression data of nine AIR-exposed and twelve CS-exposed fetuses (3 batches) were used for analysis. All samples were analysed without filtering by *ttest*, *Limma-ttest* or *Benjamini-Hochberg* for multiple testing. As we intended to generate hypotheses, we did not wish to exclude data a priori. Hierarchical Cluster Analysis was applied to probe sets with an average expression >5 , but no sex clustering was observed (Fig. 4.12). Furthermore, only a tendency to cluster according to treatment could be seen (cluster AIR-exposure: M386, M421, M187, M307; cluster CS-exposure: M191, M207, M205, F195, F201).

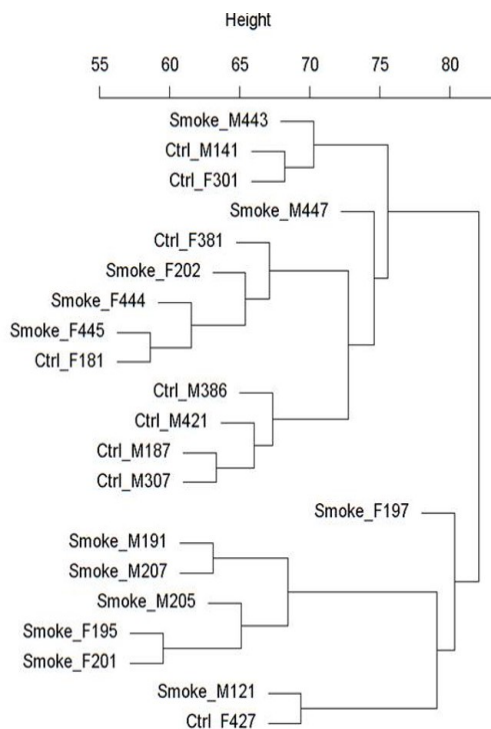


Figure 4.12: **Cluster dendrogram of pulmonary mRNA expression data** Hierarchical cluster analysis was conducted based on probe sets with an average expression > 5 . The horizontal axis (height) represents the distance or dissimilarity in expression pattern between the individuals. Nine AIR- (Ctrl) and twelve CS- (SMOKE) exposed female (F) and male (M) fetuses were included.

Based on these findings, no stratification for sex was done in following pathway analysis. From 35916 murine probe sets on the gene chip 26098 could be mapped by Ingenuity pathway analysis (IPA). For further calculations a $|\text{fold-change}| > 1.2$ and raw p-value < 0.05 was chosen. By using these restrictions 1340 genes were found to be regulated and from this gene set 1003 could be mapped by IPA. The core analysis modest of IPA was used to identify causal relationships within the regulated gene set. This core analysis is based on associations reported in the literature and which are likely relevant for the underlying biological mechanisms. In this analysis direct and indirect relationships as well as data gained from experiments with endogenous chemicals were included. Within our data set deregulated genes showed significant association with several diseases and biological functions. Table 4.2 shows the top 5 associations for three functional groups.

Table 4.2: **Top diseases and bio functions associated with changes in global mRNA expression patterns**

Diseases and Disorders		
Name	p-value	# Molecules
Cancer	4.96E-09 - 1.68E-03	582
Organismal Injury and Abnormalities	4.96E-09 - 1.67E-03	395
Reproductive System Disease	4.96E-09 - 1.26E-03	314
Respiratory Disease	1.65E-09 - 1.68E-03	142
Gastrointestinal Disease	3.22E-09 - 1.68E-03	490
Molecular and Cellular function		
Name	p-value	# Molecules
Cell cycle	8.44E-17 - 1.68E-03	178
Cellular Assembly and Organization	6.05E-15 - 1.67E-03	158
DNA Replication, Recombination and Repair	6.05E-15 - 1.67E-03	115
Cell Death and Survival	1.35E-08 - 1.09E-03	271
Cellular Growth and Proliferation	4.09E-08 - 1.08E-03	275
Physiological Systems and Development and Function		
Name	p-value	# Molecules
Organismal Survival	1.77E-07 - 1.89E-05	214
Embryonic Development	2.15E-06 - 1.67E-03	45
Organ Development	2.15E-06 - 1.67E-03	34
Organ Morphology	2.15E-06 - 1.68E-03	30
Organismal Development	2.15E-06 - 1.67E-03	123

As fetuses are still during their developmental stage, we were mostly interested in molecules regulated within the functional annotation "Physiological System Development and Function". Interestingly, several subfunctions in this group belong to functional annotations related to lung and body development (e.g. abnormal morphology of lung, size of body, morphology of lung cells; Tab.4.3). In a further analysis done by a colleague (Stefan Dehmel; upstream regulator analysis; IPA; see supplementary data) and in consensus with our phenotypic data (body and lung growth alterations) and data in the literature, *Insulin-like growth factor 1 (Igf1)* was found to be the most abundant gen which was upregulated in our data set. Additionally, its corresponding binding protein (*Igf1bp3*) was down-regulated within the data set.

Table 4.3: Physiological system development and function associated with changes in global expression patterns

Disease and Functions Annotations	p-value	z-score	Molecules
abnormal morphology of lung	2.14E-06		↓ABCA3, ↓ATP2A1, ↓CEBPA, ↓CFTR, ↑CRH
formation of embryoblast	4.22E-06		↑FEN1, ↑GMNN, ↑MYBL2, ↑NCAPG2
abnormal morphology of pulmonary alveolus	7.67E-06		↓ABCA3, ↓CEBPA, ↓CFTR, EDN2, ↓ELN
size of body	3.27E-05	-0.761	↓ABHD5, ↓ADRB3, ↓ANGPTL4, ↓AQP5
morphology of lung cells	1.38E-04		↓ABCA3, ↓CEBPA, ↑CRH, ↑GPR116, ↑IGF1
abnormal morphology of blastocyst	2.19E-04		↑AURKA, ↑C3, ↑CDK1, ↑E2F8, ↑ESRRB
abnormal morphology of interalveolar sept	2.53E-04		↑CRH, ↑HAT1, ↑IGF1, ↑PROC, ↑ROBO1
formation of lung	3.03E-04	0.00	↓ABCA3, ↓ATP2A1, ↓CEBPA, ↓CFTR, ↑CRH
mass of organism	4.17E-04	0.116	↓ACE, ↓ADRB3, ↑BRCA2, ↑C3, ↓CD36
abnormal morphology of dilated gall bladder	8.71E-04		↓CCKAR, ↓CFTR, ↓THBS1
formation of trabecular bone	1.37E-03		↑BMP3, ↑IGF1, ↓IGFBP3, ↑POSTN, ↑PTH1R
morphology of pneumocytes	1.67E-03		↓ABCA3, ↓CEBPA, ↓GPR116, ↑IGF1, ↓SFTPB

4.3.2 Validation of gene expression data

Only about 5% of the secreted IGF1 is unbound. The majority of IGF1 is bound to its counterpart IGFBP3 and within this complex IGF1 is transported to the target organ through the circulation (Butler and LeRoith, 2001). However, only unbound IGF1 can bind to its receptor (IGF1R) and induce phosphorylation of the receptor, which is essential for further downstream signaling to activate cell proliferation or prevent cell apoptosis. Thus, besides IGF1 also IGFBP3 and IGF1R are important proteins to describe the insulin-like growth factor system. Therefore, expression level of *Igfbp3* and protein amount of *Igf1R β* were analysed.

As depicted in Figure 4.13 a significant increase in *Igf1* was confirmed by qRT-PCR after *in utero* CS-exposure on E18.5 (fc=1.3, p<0.05). However, there was no effect on *Igf1* in males. Furthermore, *Igf1* expression was not altered at PND21 and at PND56. mRNA expression of *Igfbp3* is shown in Fig. 4.13 D-F. *Igfbp3* mRNA was significantly decreased only in CS-exposed females at E18.5 (fc=-1.6, p<0.05) but not in males nor at the other time points.

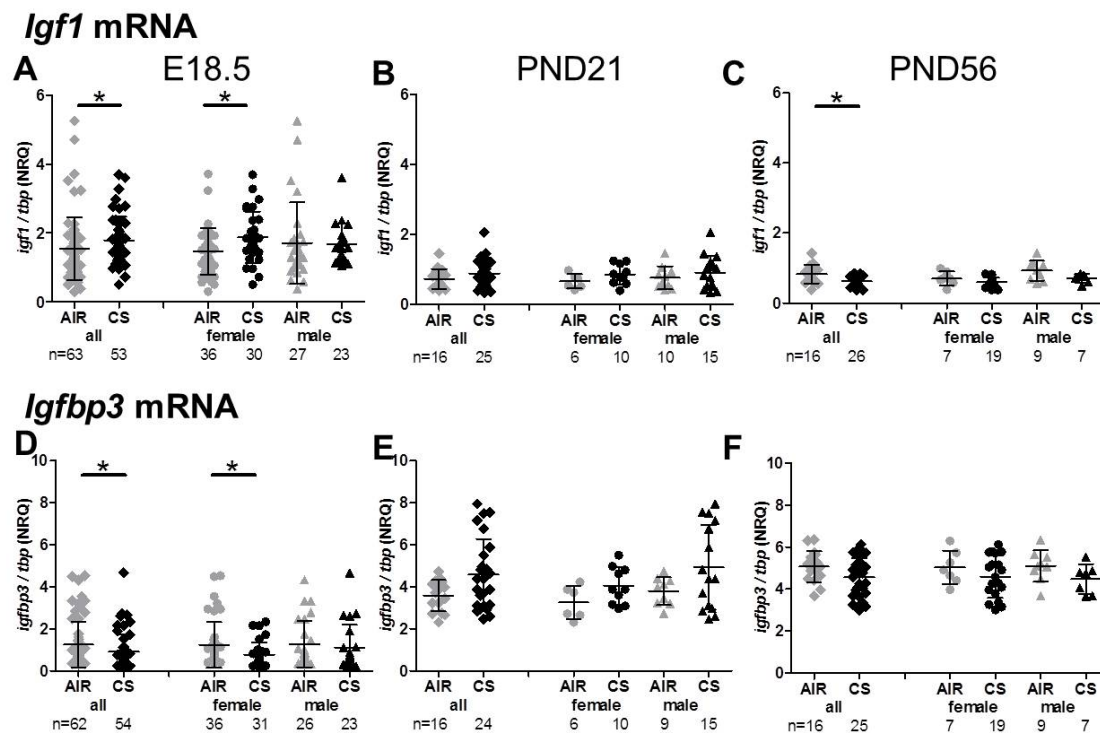


Figure 4.13: **Pulmonary expression data of *Igf1* and *Igfbp3*** Expression levels of *Igf1* (A-C) and *Igfbp3* (D-F) are represented as normalized values to the reference gene (*Tbp*). Pups after intrauterine CS-exposure (black) or AIR-exposure (gray) at three different developmental time points (E18.5 [A,D]; PND21 [B,E] and PND56 [C,F]) Circles (\circ), triangles (Δ) or diamonds (\diamond) indicate females, males or both. $n \geq 6$ (≥ 3 batches). Statistical analyses were performed by Mann-Whitney-U test with * $p < 0.05$, ** $p < 0.01$

4.3.3 Protein quantification of IGF1 at three distinct time points

For further validation of the microarray results at the protein level, Western blot analysis was performed for IGF1. IGF1 protein level was significantly decreased in males with an $fc = -1.4$ at E18.5 (Fig. 4.14). On PND21 protein amount was found to be significantly elevated after CS-exposure in both sexes on PND21 (female: $fc = 2.0$, $p < 0.05$; male: $fc = 1.5$, $p < 0.05$). In adult animals (PND56) no differences could be detected. IGF1 protein is 7.5 kDa in size. Western blot analysis revealed bands at 25 kDa (IGF1-trimer) and 15 kDa (IGF1-Dimer; 4.14G). Those bands were consequently and reproducibly found in all Western blot analysis and confirmed by the company that produced this antibody. For calculation of the IGF1-

by Western blot at PND56.

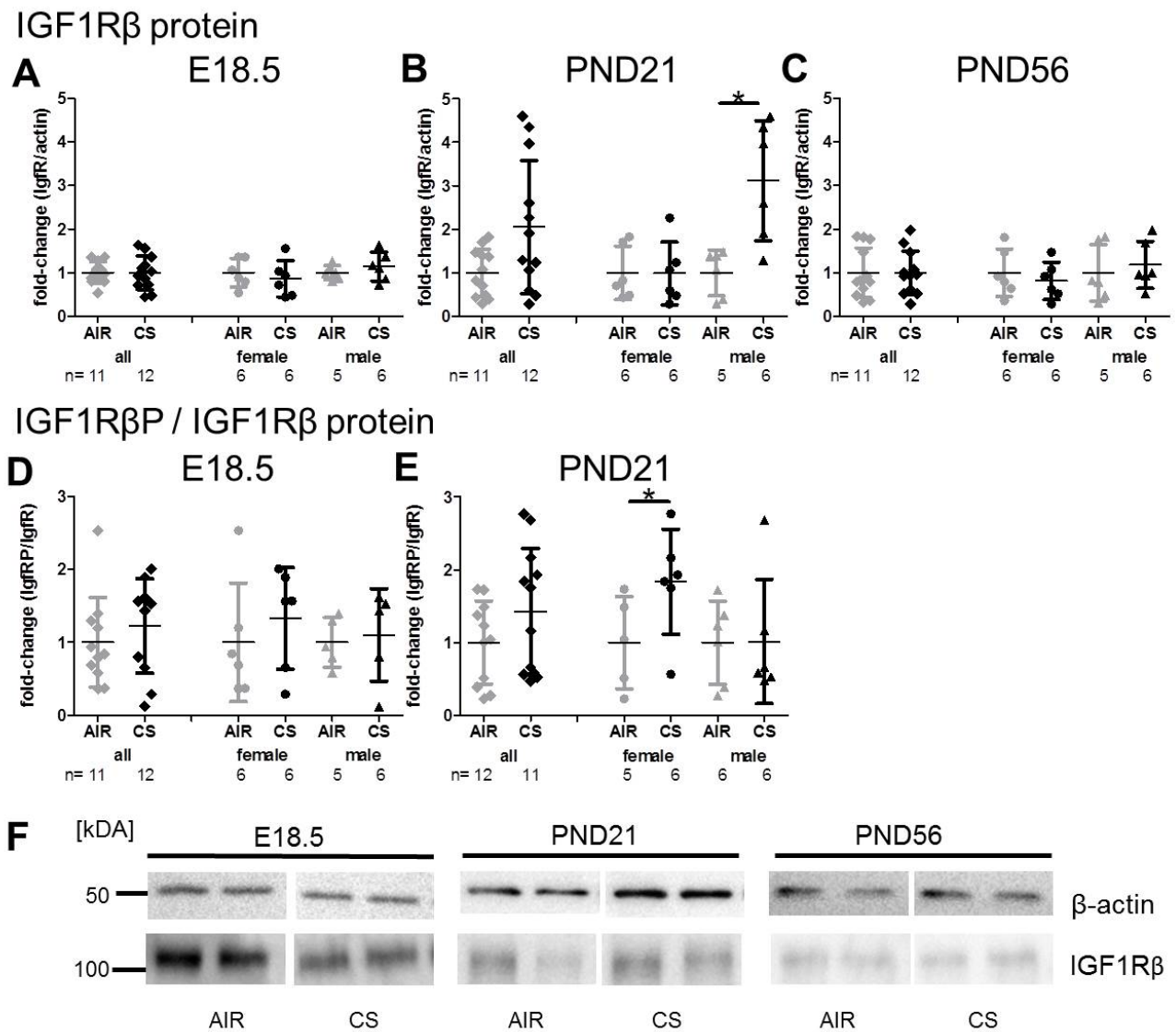


Figure 4.15: **Protein level of IGF1-receptor (IGF1R β) and its activated form IGF1R β P** Protein data are normalized against β -ACTIN as reference protein and is expressed as fold-change according to the control (AIR). For quantitative assessment of the activation, the ratio between IGF1R β P and IGF1R β was calculated [D, E]. Pups after intrauterine CS-exposure (black) or AIR-exposure (gray) at three different developmental time points (E18.5 [A]; PND21 [B] and PND56 [C]) and representing Western blots (G). $n \geq 5$ (4 batches). Circle (\circ), triangle (Δ) or diamond (\diamond) indicate females, males or both. Statistical analyses were performed using Mann-Whitney-U test (* $p < 0.05$)

4.4 Epigenetic regulation by *in utero* CS-exposure

4.4.1 Global histone modification pattern

The packaging of the DNA highly influences the gene transcription. Posttranslational modifications of the histone tails can alter the packaging of the DNA and also affects binding of effector proteins. To analyse global histone modifications in the fetuses after intrauterine CS-exposure, quantitative mass spectrometry was performed. To this end, histones H3 and H4 were isolated from whole lung tissue at E18.5. Two batches (each three CS-exposed and three AIR-exposed pups) were independently analysed.

Results of histone modification analysis are shown in Figure 4.16. No effect of *in utero* CS-exposure on global H3 modifications was found. Furthermore, no effect of intrauterine CS-exposure was measured in H4 modifications (data in appendix).

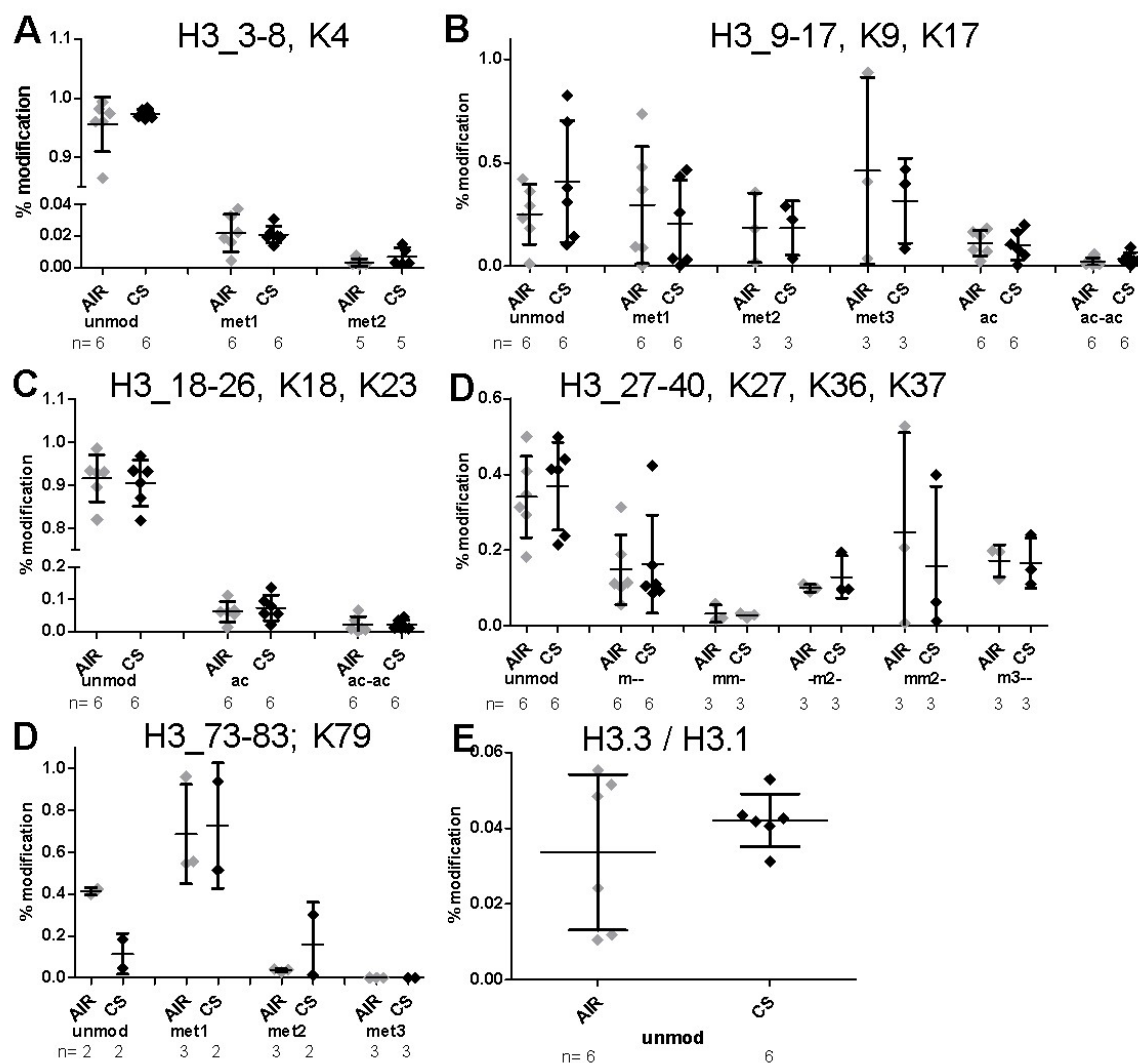


Figure 4.16: **Quantitative analysis of histone modification pattern in fetuses after in-utero CS-exposure** H3 histone of whole lung tissue were isolated. Fragments for mass spectrometry were generated by propionylation (protection of lysine-residues [K]) and further digestion by trypsin, leading to fragments: $H3_3 - 8, K4$ [A]; $H3_9 - 17, K9, K17$ [B]; $H3_{18} - 26, K18, K23$ [C]; $H3_{27} - 40, K27, K36, K37$ [D]; $H3_{73} - 83, K79$; $H3.3/H3.1_{27} - 40$ [E]. Diamonds (\diamond) indicate females and males. $n \geq 2$ (≥ 2 batches). Statistical analysis was done by Mann-Whitney-U test

4.4.2 Local histone modification pattern

As *Igf1* expression was affected by *in utero* CS-exposure, we asked, if local histone modifications at the *Igf1*-promoter might be changed. Therefore, we analysed activating (H3K4met3)

and repressing (H3K27met3) histone marks by Chromatin-Immunoprecipitation (ChIP) at the *Igf1*-promoter (Fig.4.17). Methylation of H3K4met3 was not altered between CS- and AIR-exposed fetuses. However, the amount of H3K27met3 was trendwise decreased after intrauterine CS-exposure in females ($p=0.09$). No effect on males was measured.

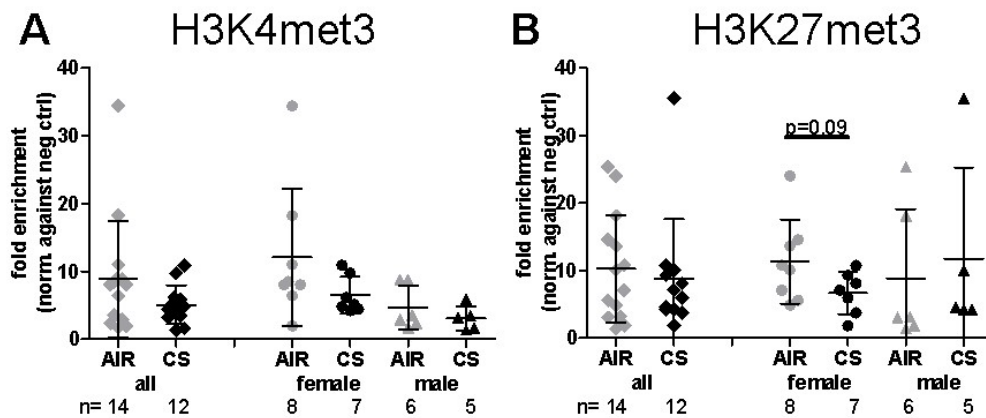


Figure 4.17: **Chromatin-immunoprecipitation of *Igf1* promoter** H3K4met3 and H3K27met3 modification were enriched by ChIP with the *Igf1* promoter in E18.5 fetuses and the quantification of the product was done by qRT-PCR of the promoter region against a negative control (IgG antibody). Circles (\circ), triangles (\triangle) or diamonds (\diamond) indicate females, males or both. $n \geq 5$ (≥ 2 batches). Statistical analysis was done by Mann-Whitney-U test

Chapter 5

Discussion

Within this study the effect of intrauterine CS-exposure on the development of murine offspring was investigated on three distinct developmental time points (E18.5 [fetal stage], PND21 [adolescent stage] and PND56 [adult stage]). For this purpose, phenotypic data such as body weight, lung weight as well as lung function analysis and airway remodeling were assessed. Fetal body and lung weight were significantly decreased after *in utero* CS-exposure in female pups. Subsequently, body weight in growing offspring was significantly lowered after *in utero* CS-exposure independent of sex. Lung function analysis revealed a CS-related decrease in inspiratory capacity per body weight in both sexes and an increased airway hyperreactivity (AHR) after metacholine challenge in females at PND21. These alterations were not observed on PND56. Analysis of the pulmonary fetal mRNA expression profiles revealed that *Igf1* was significantly upregulated in intrauterine CS-exposed offspring. Further validation by qRT-PCR confirmed those data. However, fetal protein levels of IGF1 were significantly lowered in CS-exposed offspring, whereas in adolescent animals significantly higher IGF1 levels were observed. Furthermore, histone modification analysis revealed a trend to reduced methylation at the lysine 27 at histone 3 (H3K27met3; repression mark) at the *Igf1*-promoter in female fetuses after intrauterine CS-exposure.

5.1 Impact of cigarette smoke on pregnancy related parameters

This study aimed to establish a moderate cigarette smoking protocol, that would not harm the normal course of pregnancy. To ensure that our protocol mirrors human smoking behaviour, cotinine levels and CO-hemoglobin amounts were assessed. Furthermore, we analysed basic maternal parameters like weight gain, pregnancy duration and litter size to assure that observed effects in the offspring are caused by intrauterine CS-exposure and not by a compromised pregnancy.

5.1.1 Cigarette smoke exposure protocol

So far, many murine inter- and transgenerational smoke exposure models are published, which differ in type of exposure, term of exposure and amount of exposure. Regarding type of exposure, some models aimed to simulate passive smoking(= sidestream smoke; (Penn et al., 2007; Rouse et al., 2007)), active smoking (Blacquière et al., 2009; Singh et al., 2003) or used nicotine as surrogate (Liu et al., 2011; Rehan et al., 2012). In terms of exposure preconceptual (Blacquière et al., 2009; Singh et al., 2003), prenatal (Larcombe et al., 2011) and postnatal (Manoli et al., 2012) (Wu et al., 2009) is applied. Furthermore, the amount of exposure (times per day and number of cigarettes) varies highly. Also, prenatally exposed offspring might be challenged with allergens (Singh et al., 2011; Blacquière et al., 2009) [Fig.6.4]. Most of these studies mentioned above did not include CO-Hb or urine cotinine measurements to validate the CS-exposure. Only Larcombe et al (2011) measured urine cotinine within their model which consists of mainstream cigarette smoke on second and third trimester of murine pregnancy (GD 8 to GD 20) with three cigarettes twice per day. The measured urine cotinine amount of 5799 ng/mL in CS-exposed dams was 8-fold higher than the cotinine level reached with our protocol. However, we took urine samples 4 h after the last cigarette, while Larcombe et al (2011) collected the urine directly after the last exposure.

Within our study, CO-Hb and urine cotinine values of CS-exposed animals were comparable to human light to moderate smokers (<1 pack/day; (Schane et al., 2010)) (Matsumoto et al.,

2013; Hampson and Scott, 2006). According to Chechowska et al (2013) the amount of serum cotinine correlates with the amount of daily cigarette consumption in humans. Thus, we hypothesized that the murine CS-exposure model established within this study is comparable to human mild to moderate smokers. Several studies have already shown that smoking during pregnancy can affect placental function, which might result in mal nutrition of the fetus (Stone et al., 2014) and oxidative stress of the placenta (Garrabou et al., 2014). However, the model presented in this work does not allow to distinguish between direct effects of cigarette smoke on the offspring and side effects caused by stress or by alterations in placental function through cigarette smoke consumption.

5.1.2 Influence of CS on course of pregnancy

The gain of body weight during pregnancy of CS-exposed females was significantly reduced compared to AIR-exposed mice. Despite this effect, other pregnancy parameter such as pregnancy rate, pregnancy duration, litter size and amount of visible resorptions were not significantly altered by CS-exposure within this model. In a comparable murine study, pregnant mice were exposed to mainstream CS for 4h/day on 5 day/week with a CO-Hb level of 25 ppm (human: <1 pack/day) which led to reduced gestational length (0.7 days) in CS-exposed mice. According to the authors, this reduction was equivalent to a 1.3 week reduction of gestation in humans indicating a toxic effect of intrauterine CS-exposure causing preterm delivery (Ng et al., 2006). Our model did not reduce the gestational length. Also Larcombe et al. (2011) described no effect of intrauterine CS-exposure on the litter size, yet the low number of investigated pups has to be taken into account (AIR: 3 dams; CS: 4 dams). So far, a detailed analysis of pregnancy outcome in relation to cigarette smoke exposure has been poorly investigated in the literature. Carmines et al. (2003) exposed rats to mainstream CS-exposed and reported a reduced weight gain during pregnancy at particle concentrations of 600 TPM/ m^3 . Other gestational parameters like litter size and resorptions per litter were not affected. However, pregnant rats showed a clinical phenotype with diarrhea, salivation and red eyes caused by CS-exposure. Those effects were not observed in our study. Along this line it has been reported that smoking pregnant woman, gained about 5% less weight than non-smoking women (Groff et al., 1997).

5.2 Murine phenotypic alterations of the offspring are comparable to observations in humans

It has been proposed in the literature that environmental exposures during fetal development can affect body weight, lung growth and developmental processes (Gilliland et al., 2001; DiFranza et al., 2004). In humans these alterations can lead to intrauterine growth restriction (IUR) and preterm birth (Hancox et al., 2009). Additionally, it has been shown that prenatal CS-exposures results in impaired lung function and increased susceptibility for chronic airway disease such as asthma and COPD (Hancox et al., 2009; Gluckman et al., 2005).

5.2.1 Impact of litter size

When comparing murine with human studies the litter size is a major challenge (Priestnall, 1972). Naturally, mice have a litter size between two to ten pups. For this reason phenotypic data of the pups might differ as nutrient supply per pup will be different. In order to reduce this confounding factor, fetal phenotypic data were stratified for litter size (litter size >6 and litter size ≤ 6) and body weight. Further regression analyses was corrected for sex and litter size.

5.2.2 Fetal phenotype

Lung weight and lung/body weight ratio after intrauterine CS-exposure were significantly decreased in female fetuses but there was no obvious effect on males (litter sizes >6). Reduced body weight has already been reported in studies of murine offspring after intrauterine CS-exposure, but lung weight was not analysed (Larcombe et al., 2011).

These results are in line with human studies (Benjamin-Garner and Stotts, 2013; Caudri et al., 2007), which described an association between maternal smoking during pregnancy and low birth weight. However, a recent meta-analysis in humans by Sonnenschein-van der Voort et al. (2014) proposed that preterm birth caused by intrauterine CS-exposure is the

reason for low-birth weight within those children. Regarding sex differences, female lungs mature earlier than males, which could be observed by mouth movements around gestational week 20 that reflect fetal breathing (Hepper et al., 1997). This indicates an earlier surfactant production in female fetal lungs (Fleisher et al., 1985), showing that there are sex differences in lung development that might explain the higher influence of intrauterine CS-exposure in females.

However, one should bear in mind that nicotine, which is the main psychoactive component in cigarettes, interacts with nicotinic cholinergic receptors (nAChR) and leads to an increase in energy expenditure. In humans it is well known, that nicotine perfuses the placenta resulting in a 15% higher nicotine concentration in the fetal than in the maternal circulation (Eskenazi et al., 1995; Triche et al., 2008). Therefore, nicotine might lead to increased energy turnover in the fetal organism and could possibly lead to the significantly reduced weight gain after CS-exposure (reviewed in Shea and Steiner, 2008).

5.2.3 Body weight analysis in growing up offspring

In addition to the results obtained in fetuses, body weight analyses were performed from PND2 until PND59. Both sexes showed significantly reduced body weight gain in *in utero* CS-exposed pups over time. Of note, the number of animals included in weight analysis at later time points (PND 28-59) is relatively small compared to number of pups analysed in earlier developmental stages, thus reducing the statistical power of later time points. So far, to our knowledge, there is no other study in mice that analysed long term body weight progression after intrauterine CS-exposure. A number of studies using *in utero* nicotine-exposed rats showed controversial data regarding long-term body weight development (reviewed in Abbott and Winzer-Serhan, 2012). The authors conclude that other environmental factors (e.g. housing conditions, maternal behavior) significantly influence postnatal development as well, indicating that it is very difficult to restrict causation to CS.

In the present study a delayed body weight gain was observed between PND15 to 21. This periode corresponds to the end of weaning and the start of individual food intake of the offspring. Along this line, human cohort studies showed a CS-related delay in growth only in

early childhood with later catch-up growth (Kannelopoulos et al., 2007; Kotecha et al., 2010). There are also controversial reports in the literature (Anand et al., 2003).

5.2.4 Lung function measurements after intrauterine CS-exposure

In the present work, intrauterine CS-exposure resulted in reduced inspiratory capacity (after correction for body weight) in both sexes and a significantly elevated AHR in females at PND21.

A reduction of thoracic gas volume has been described in intrauterine CS-exposed pups as well, however without significance after correction for body weight (Larcombe et al., 2011). The authors further investigated lung mechanics and found elevated levels in tissue damping (G) and tissue elastance (H) in CS-exposed pups. The authors speculate, that this might be caused by impaired development and lung size. Similarly, an increased AHR after smoke exposure has been shown by Blacquière et al. (2009). A rat model of intrauterine nicotine exposure reported increased pulmonary resistance without metacholine challenge and suggested that the interaction between nicotine and its receptor (nAChR) in developing lungs might be responsible for alterations in lung mechanics (Sekhon et al., 2001). Pike et al. (2014) claimed changes in airway smooth muscle after in *in utero* protein restriction diet as possible cause for hyperresponsiveness in isolated rat bronchi. Lung mechanics (elastance, damping) or tissue remodeling might be also involved in the observed reduction in I_c/wt after CS in the present study. However, the observed AHR might also be explained by an imbalance in nutrition due to alterations in placental function after CS (reviewed by Shea and Steiner, 2008).

Studies in school-age children reported a significant association of intrauterine CS-exposure and reduced lung function, especially in small airway flows (Gilliland et al., 2000, 2001; Svanes et al., 2004). Furthermore, newborns (4.2 weeks old) already had a significantly reduced lung function (FEV1) after *in utero* CS-exposure (Hanrahan et al., 1992). In summary, various reports in the literature and our own data suggest that prenatal maternal smoke exposure affects the *in utero* airway development and alters lung elastic properties.

5.2.5 Analysis of airway remodeling in the offspring of CS-exposed mice

In order to further investigate if lung function deficits can be explained by airway remodeling, smooth muscle and collagen were analysed on mRNA and protein level. Both proteins are part of the extracellular matrix and their deposition leads to a thickened airway wall and markedly reduced airway caliber resulting in increased resistance to airflow and bronchial hyperresponsiveness (Bousquet et al., 2000). Protein levels of α SMA were significantly elevated at the fetal stage which was maintained until PND21. In contrast, *Acta2* (α SMA) expression was not altered. Of note, CS-exposure did not influence the mRNA expression or protein levels of Collagen1. The elevated protein levels of α SMA especially in males might contribute to the reduction in Ic/wt which was more pronounced in male offspring at PND21, although the mRNA level was not altered.

Blacquière et al. (2009) observed increased amounts of Collagen III and α SMA in adult mice after prenatal CS and proposed the thickness of α SMA layer as indicator for AHR. However, the authors used a stronger cigarette smoke exposure (10 cigarettes, twice a day) and already started cigarette smoking of the dams preconceptionally.

An increase in remodeling proteins is consistent with experiments in *in utero* nicotine exposed rhesus monkeys where a 1.7 fold increase of collagen surrounding the airways was found (Sekhon et al., 1999). Regarding human findings, 6 month old babies showed elevated inner airway wall thickness after intrauterine and postnatal smoke-exposure, which might be based on increased collagen deposition (Elliot et al., 1998).

5.2.6 Evaluation of the established model regarding phenotypic analysis

While human data showed a clear correlation between maternal smoking and intrauterine growth restriction (IUR) as well as an increase in asthma and wheezing of children later in life (Sonnenschein-van der Voort et al., 2014; Baker et al., 1993), the results available from animal studies are not consistent regarding intrauterine cigarette smoke exposure and fetal characteristics. So far, the present, newly established murine model is the first one to

our knowledge, which shows in depth analysis of pregnancy parameters as well as offspring phenotypic data from fetal stage until adulthood. It therefore replicates the main findings made in prenatal children. Furthermore, there are hints for an IUR after smoke exposure and lung growth was disproportional more affected in female pups. The increase in α SMA deposition might in part explain the observed lung function alterations (Ic/wt and AHR) after intrauterine CS-exposure. We speculate that, the observed growth retardations after *in utero* smoke exposure might lead to a disturbed lung development and enhanced remodeling, which, in consequence, might lead to a decline in respiratory function later in life.

5.3 Pulmonary gene expression profile reveals alterations in *Igf1*

5.3.1 Fetal expression profiling of whole lung tissue

The development of complex diseases such as allergy, cardiovascular disease and diabetes has been linked to alterations in the expression of multiple genes and trait loci (reviewed in Andreassi, 2009; Tripathi et al., 2014). Nowadays, gene-environment interactions are in the research focus for their role in etiology of complex diseases (reviewed in von Mutius, 2009). As being the first environment for the developing fetus, *in utero* environment has a high impact on developmental processes in general and consequently on the development of chronic diseases (reviewed in Dabelea and Crume, 2011; Dolinoy et al., 2006).

This work studied the impact of intrauterine CS-exposure on pulmonary gene expression immediately before natural birth. When analyzing the mRNA expression profiles, no clustering according to sex and only a trend to cluster for treatment could be observed. However, these results are in contrast to our phenotypic data, where a clear sex difference in lung weight and lung/body weight ratio was observed. Within this project overall changes in mRNA expression were rather small. As a consequence, even smaller changes caused by sex might be missed. The differences in gene expression might be explained by the low-to moderate exposure protocol to avoid high toxic doses. Additionally, the tissue collection was performed 24 h after the last CS-exposure to avoid seeing acute but only transient CS-exposure effects.

These small changes within gene expression profiles might reflect the early onset of pathological changes caused by intrauterine CS-exposure, whereas stronger changes might occur at the incidence of the disease.

Suter et al. (2011) and (Breton et al., 2009) reported also no clustering according intrauterine CS-exposure within their blood and placental tissue from children. The authors suggested this to reflect multiple interactions among the pathways and genes in the subjects as possible explanation.

We aimed to identify top diseases and functions associated with changes in global mRNA expression patterns, by using the core analysis module of the Ingenuity Pathway Analysis (IPA) software. This analysis revealed that functions associated with organismal development were strongly affected by *in utero* smoking. However, we also observed the groups 'Cancer' and 'Organismal injury and Abnormalities' as groups with high p-value. This might be explained by the structure of IPA. The data basis of IPA is greatly facilitated by the consideration of prior biological knowledge, in which numerous associations between molecules, phenotypes and diseases are described (Krämer et al., 2014). As the total amount of cancer related publications is much higher than e.g. for developmental processes, IPA shows a higher reference quantity for cancer. Nonetheless, 'Respiratory disease' was one of the top hits within the underlying genes as well. Together with the group 'Physiological System Development and Function', which included functional annotation like 'Embryonic Development' and 'Organ Development', this gene expression profile provides a molecular framework which matched the observed phenotypic data (e.g. alterations in lung function and remodeling). In a further network analysis, Insulin-like growth factor (IGF1) emerged as developmental key molecule, which could be responsible for the observed phenotypic changes.

The only murine study published so far, that performed pulmonary mRNA expression profiles after intrauterine CS-exposure was conducted by Rouse et al. (2007). The authors exposed pregnant mice to sidestream smoke and analysed mRNA expression in 15 week old offspring. IPA analysis revealed that the significantly regulated genes belong mainly to groups

like cell proliferation, cell survival, cell metabolism, cytoskeletal structural components and ubiquitination/ apoptosis. These functional annotations can be grouped mainly under the term 'maintenance' of the organism, which is in relation to the age of their used animals. Our data revealed mainly groups globally assigned to 'development', besides 'Cancer' as discussed above, with regards to the fetal stage of the used tissue.

5.3.2 Pulmonary IGF1 levels are altered after intrauterine CS-exposure

As mentioned above, *Igf1* was one of the abundant genes after network analyses, although, it was not the gene with the highest fold-change and lowest p-value. This growth hormone was chosen for further analysis in a hypothesis driven way as it has been already described to correlate with birth weight in mouse and human (McIntyre et al., 2000; Woods et al., 1996; Pais et al., 2013). In the present study, the observed up-regulation of *Igf1* and down-regulation of *Igfbp3* was confirmed by qRT-PCR in fetuses, while later developmental points revealed no adverse regulation by CS-exposure. In a further validation step, IGF1 protein level were assessed by Western blot analysis showing significantly decreased IGF1 level in CS-exposed fetuses while adolescent CS-exposed pups showed significantly elevated levels.

Igf1 has been thoroughly characterized for its role in tissue development and homeostasis by activating the downstream signaling cascade through binding to the *Igf1* receptor (reviewed in Lee et al., 2014). After receptor binding a complex phosphorylation cascade activates multiple pathways including mitogen-activated protein kinases (MAPK), protein kinase B (PKA, Akt) and signal transducers and activator of transcription (STAT) are activated. This results in either cell growth and proliferation or inhibition of apoptosis. Postnatal production of *Igf1* is stimulated by growth hormone (GH) in the liver, while fetal *Igf1* is produced in many tissues in an autocrine/paracrine fashion.

The importance of the paracrine action of IGF-binding proteins in the developing lung was confirmed by Wallen et al. (1997) showing that the expression of the different *Igfbp* was cell specific whereas IGFBP peptides were widely distributed in the lung. Further, it was

shown in rats that the highest IGF1 protein expression was found in early postnatal days (PD 5-7; alveolar stage) which lead the authors to speculate about the importance of IGF1 in lung development (Liu et al., 2004). Besides the effects of IGF1 on murine lung growth, there is also evidence that *Igf1* is essential for pulmonary cell proliferation and differentiation (Narasaraju et al., 2006) as well as for lung vasculargenesis of mice (Tanswell et al., 1991). Furthermore, *Igf1*^{-/-} mice showed severe embryonic growth retardation with a postnatal death rate of 100% (Pais et al., 2013).

Accompanying the results in homozygous knock-out mice, a boy, with a homozygous deletion of the *IGF-1* gene, showed severe prenatal and postnatal growth failure (Woods et al., 1996). Experiments in human fetal and embryonic lung explants demonstrated the importance of *Igf1* on lung vasculargenesis, which is critical for optimal gas exchange after birth (Han et al., 2003). Expression levels of *Igf1* were also drastically elevated in lung malformation in human stillbirths who died because of respiratory distress syndrome [RDS] and bronchopulmonary dysplasia [BPD] (Chetty et al., 2004; Miyazaki et al., 1998). Further, several human cohort studies investigated the effect of maternal smoking on the IGF1 levels in the offspring and found it to be reduced in cord blood after CS (Ingvarsson et al., 2007; Palmer et al., 2002; Coutant et al., 2001). These data suggest an association of *in utero* CS-exposure and alterations in the *Igf1* system. Similar to the reversed expression of our mRNA and protein data on IGF1, Capoluongo et al. (2008) described a similar regulation pattern in human stillbirth after BPD. The authors observed high protein levels of IGF1 in epithelial lining fluid but low serum IGF-levels. Furthermore, in children with low birth weight, serum IGF1 levels were dramatically increased at the age of 4 years (Fall et al., 1995). This increase was even higher than expected for their height and weight. The authors claimed an adequate postnatal nutrition situation or a postnatal increase in growth hormone (GH), which modulated *Igf1* action, as possible cause for the IGF1 increase. The observed differences in mRNA expression versus protein level might further be explained by different regulatory patterns between transcription and translation like half-life of mRNA, translation rate ect.

5.3.3 IGF1 downstream signaling

Igf1 acts primarily through its high affinity receptor IGF1R, which results in phosphorylation of the β -subunit and leads to an initiation of the downstream signaling cascade. Therefore, also levels of IGF1R and its activated (phosphorylated) form are essential for growth and lung development. Within our model, IGF1R was significantly up-regulated in males at PND21 with no change at other developmental points. The receptor activation was also more pronounced at PND21 in female pups. The lack of changes in IGF1RP/IGF1R in males at PND21 might be due to higher receptor protein levels per se, masking the change in phosphorylated receptors.

The importance of the IGF1R has been shown in IGF1R knock-out mice, which died shortly after birth from respiratory distress and exhibit pronounced growth deficit (Liu et al., 1993; Epaud et al., 2012). Furthermore, alterations in the *IGF1R* gene are associated with growth effects and IUR or lung hyperplasia in humans as well (Kruis et al., 2010). A deletion in chromosome 15 was found in an infant with severe IUR and lung hypoplasia. The deleted region could be specified as gene region of *Igf1r* and validated by qRT-PCR as a lost copy of *Igf1R*.

5.3.4 Conclusion of pulmonary expression data

Lung development in mice sprawls into adolescence with the development of the alveolar ducts from E17.5 until PND5, and is finished at PND30 with the alveolar stage. Thus, the Igf1-axis seems not only to be essential for prenatal murine lung and growth development but also during postnatal lung development. Based on our data, we speculate that *in utero* CS-exposure induces a delay in Igf1 protein expression. This delay is abrogated after birth and leads to a postnatal overproduction of IGF1, which might also explain the intrauterine growth retardation observed in our model. The alterations in AHR and remodeling markers of the lung could also be influenced by IGF1 alteration (reviewed in Lee et al., 2014). In line with these findings, OVA-challenged mice that were treated with an Igf1-antibody to counteract the Ova-induced up-regulation of pulmonary *igf1* expression, showed increased AHR, airway inflammation and airway wall thickening (Yamashita et al., 2005). Further-

more, *in vitro* experiments have shown that IGF1 increases smooth muscle proliferation and induces SMA contractions. Hoshino et al. (1998) and colleagues analysed bronchial biopsies from asthmatic patients and revealed an association between *Igf1* expression and collagen thickening. The authors concluded that modulation of *Igf1* expression might inhibit inflammatory cell infiltration in the airways and therefore, might influence remodeling processes. Our model suggests that intrauterine CS-exposure results in deregulation of *Igf1* expression as well as of IGF1 protein levels, which may cause reduced neonatal lung weight resulting in lung function deficits in adolescent mice. Deregulation of the growth hormone axis by prenatal smoke exposure might be one cause of the observed paediatric phenotype with growth restriction and lung function deficits. Furthermore, we speculate, that deregulated *Igf1* affects remodeling processes which leads to increased smooth muscle deposition and may explain observed AHR after intrauterine CS-exposure.

5.4 Epigenetic changes by intrauterine cigarette smoke exposure

Epidemiological evidences suggests that early life exposures can be passed on to subsequent generations. The change of epigenetic marks is proposed as a basic mechanism by which prenatal exposure could be linked to phenotypic alterations in adulthood. These marks are reversible, can be inherited (long-lasting effects) and cause changes in gene expression that can modulate disease susceptibility (reviewed in Jirtle and Skinner, 2007). Epigenetic changes involve a number of mechanisms including chromatin modifications and DNA modifications. Histone modifications patterns were investigated within our model to analyse a possible epigenetic regulation of the *Igf1* promoter.

5.4.1 Histone modification pattern

Local histone modifications We aimed to investigate specific histone modifications at the *Igf1* promoter sequence as *Igf1* mRNA levels were elevated in CS-exposed fetuses. This could be due to increased transcription or reduced mRNA degradation of the *Igf1* gene. An

elevated transcriptional rate might be caused by altered histone modifications at the promoter site. Therefore, common histone marks for actively transcribed genes (H3K4met3) and for developmentally repressed gene loci (H3K27met3) were investigated by ChIP experiments in whole fetal lung. No differences in H3K4met3 were observed between both groups. However, there is a trend to reduced levels of the trimethylated form of H3K27 in *in utero* CS-exposed females. Within this context, reduced repression (H3K27met3) of the *Igf1* gene might result in the observed increased in *Igf1* mRNA level at E18.5.

Reduced IGF1 protein combined with decreased *Igf1* mRNA expression profile in growth retarded animals was also presented in a food-restriction IGF1-study in rats (Fu et al., 2009). This mirrors our results and suggests that both systems are highly influenced by environmental factors. The authors performed a number of ChIP experiments along the histone 3 of the hepatic *Igf1* gene to investigate changes in a gene site-, time- and sex-dependent manner. Beside other modifications, at postnatal day 21, males showed a significantly elevated levels of H3K4met3 at the 3'UTR of *Igf1* while females showed retarded methylation at H3K36 at *Igf1*. Despite the fact, that serum *Igf1* levels were decreased, *igf1* expression depending on the lysine, which was analysed, was not altered. The authors concluded that the transcriptional elongation process was changed by altered histone marks and results in diminished levels of *Igf1*. In a follow-up study of the same group, intrauterine food-restricted newborn rats (PND1) had decreased liver weights associated with reduced H3K4met2 (activating mark) at the *Igf1* promoter (Tosh et al., 2010). However, these analyses were performed in liver tissue. Thus, *Igf1* could be regulated in a different way than lung development. Furthermore, the intrauterine stress differs from our *in utero* CS-exposure protocol. Both models affected early developmental stages and resulted in growth retardation in the offspring and alterations of *Igf1*, probably induced by a changed histone code. In a study conducted by Rehan et al. (2012) acetylation in H3 and H4 were analysed after *in utero* nicotine exposure in rats. Besides an asthma-like phenotype, a significantly increased acetylation of H3 in lungs and testes of the F1 offspring was observed. This is another hint that also acetylation of histones might play a role in lung development.

Global histone modifications After analysing acetylation and methylation pattern in H3 and H4 fragments of whole lung tissue no difference between intrauterine CS- to AIR-exposed offspring could be detected.

As already discussed in our mRNA data, the chosen protocol is rather mild-to-moderate. Thus, we expected only minor changes in epigenetic marks. It is well known that histones are modified in a tissue- and cell-specific manner (Cedar and Bergman, 2009), and the use of lung homogenate as mixture of various cell types might therefore mask possible regulation. Besides the pulmonary global histone modification pattern, there might be an alteration in other tissues or fluids caused by intrauterine CS-exposure. Furthermore, within this study whole lung tissue was analysed. This includes the different pulmonary cell types (airway epithelial cells ATII, ATI, epithelial cells, mesenchymal cells). Thus, histone modifications might be disguised by differential regulation in each cell type (Chun et al., 2015). The focus on a special cell type within the neonatal lungs might reveal more information regarding histone modifications by *in utero* CS-exposure in the future.

5.5 Summary

In humans, prenatal smoking has been related to low birth weight in the offspring. Furthermore, prenatally exposed children show lung function alterations until preschool years. Cohort studies revealed that lung function either tracks or declines but never improves later in life. Additionally, *in utero* cigarette smoke exposed children showed a higher prevalence of chronic obstructive airway disease such as asthma and COPD later in life, which might result from reduced lung function. For both diseases only symptomatic medication is available while curative therapies are lacking. Thus, there is an urgent need to develop novel and effective therapies and preventive strategies. Although prenatal smoking is well known to negatively affect the offspring, the underlying mechanisms of the disease development are largely unknown. Epidemiological studies indicate a transgenerational transmission of disease risk that might contribute to the rises of chronic airway diseases during the past decades. Further, it has been shown that environmental exposures can affect the regulation of essential genes at developmentally critical points. The emerging field of epigenetic mechanism may provide a link between early developmental exposures and disease susceptibility and therefore, might allow intervention and prevention strategies.

The present study aimed to establish an animal model of prenatal exposure to active maternal smoking in order to be able to investigate the underlying mechanisms. The phenotype of our smoke exposed offspring closely resembled observations made in prenatally exposed children: First, birth weights were significantly reduced. Second, fetal lung growth was more affected in CS-exposed females pups. Third, lung function deficits were found after intrauterine CS-exposure in both sexes. Fourth, intrauterine CS-exposure seems to increase pulmonary remodeling processes (α -smooth muscle actin and collagen deposition). Therefore, the established murine model appeared to be suitable to investigate molecular alterations involved in the phenotypic development caused by intrauterine CS. The growth hormone *Igf1* was significantly reduced after *in utero* cigarette smoke exposure in fetal lung. Epigenetic studies revealed a non-significant trend for decreased histone 3 tri-methylation at lysine residue 27 (H3K27met3) at the *Igf1* promoter site in whole lung tissue. This histone mark is associated with silencing processes of developmental genes. However, in this context, reduced tri-methylation at H3K27 might result in an increased mRNA expression of

the underlying gene. Global histone modification pattern were not affected upon intrauterine CS-exposure.

In conclusion, the results suggest that prenatal smoking deregulates pulmonary growth hormones and these alterations might be associated with impaired lung function in the absence of an allergen challenge in adolescents. These results imply that deregulated pulmonary growth hormones may link prenatal cigarette smoke exposure to increased pulmonary disease susceptibility. This established model, is the first murine model with in depth analysis of the outcome of CS-exposure during pregnancy on the maternal and fetal site. Furthermore, the offsprings phenotype was investigated longitudinally which allows comparison to human situation from the newborn until adulthood. This study suggesting a direct association between intrauterine CS-exposure, postnatal lung function deficits and alterations in a growth hormone which might be caused by changes of the histone code. However, our model allows not to separate effects of intrauterine growth retardation and prenatal CS-exposure on the lung. Although no difference was observed in pregnancy related parameters, we can not exclude that placental function was affected by CS-exposure. Furthermore, CS-exposure might be an additional stress factor which influences pregnancy, which can not be counteracted by similar handling of control and CS-exposed mice.

A number of questions remain open: Does CS-exposure alter the downstream signaling (MAPK, AKT or STAT)? Can the deregulation of IGF1 by CS-exposure passed on to the next generation (F2) without additional exposure of F1? Is DNA methylation altered by CS-exposure? Can the diminished methylation on H3K27 which was observed in this model be increased by histone-methyltransferases (HMTs) and therefore increase the *Igf1* expression *in utero*? Thus, further studies are needed to further understand the mechanisms underlying lung function deficits caused by prenatal smoking.

5.6 Zusammenfassung

Die Hauptursache von geringem Geburtsgewicht bei Neugeborenen ist das Rauchen der Mutter während der Schwangerschaft. Diese pränatal exponierten Kinder zeigen zudem eine verringerte Lungenfunktion im Vorschulalter. Die maximale Lungenfunktion wird etwa im Alter von 22-24 Jahren erreicht. Epidemiologische Studien zeigten, dass das Lungenfunktionsmaximum entweder konstant schlecht bleibt oder sogar abnimmt, sich jedoch nicht verbessern wird im weiteren Verlauf des Lebens. Weiterhin wurde belegt, dass die Prävalenz von chronisch obstruktiven Lungenerkrankungen, wie Asthma und COPD, in Kinder von rauchender Müttern deutlich erhöht ist. Dieser erhöhte Prävalenz ist möglicherweise auf eine verringerte Lungenfunktion zurück zu führen. Bis jetzt gibt es für oben genannte Lungenerkrankungen nur eine medikamentöse Behandlung der Symptome jedoch keine Heilung. Somit ist die Entwicklung neuer Behandlungsmöglichkeiten unabdingbar. Ein besonderes Augenmerk liegt dabei auf präventiven Therapien. Insbesondere für präventive Massnahmen müssen jedoch die zugrunde liegenden Mechanismen bekannt sein. Studien im Rahmen humaner Kohortstudien zeigten, dass die Exposition mit verschiedenen Umweltfaktoren in frühen Entwicklungsstadien (pränatal und postnatal) mit einem deutlich erhöhten Risiko für chronische Lungenerkrankungen im Erwachsenenalter einhergeht. In diesem Zusammenhang wurden verschiedene Faktoren wie elterliches und grosselterliches Rauchen, elterliche Allergie und Ernährung während der Schwangerschaft aufgeklärt. Die direkten Zusammenhänge sind jedoch unbekannt. Die Beobachtung, dass nicht nur die Kinder (F1) sondern auch die Enkel (F2) ein erhöhtes Risiko für Lungenerkrankungen nach mütterlichem Rauchen zeigen, deutet auf eine inter- und transgenerationale Weitergabe des Krankheitsrisikos hin. Da eine geringe Exposition von Umwelteinflüssen nicht direkt zu einer Mutation der zugrundeliegenden Gensequenz führt und damit den Anstieg an Lungenerkrankungen erklären würde, werden epigenetische Mechanismen hinter diesem Anstieg vermutet. Das Gebiet der epigenetischen Mechanismen stellt eine Verbindung zwischen Exposition in frühen Entwicklungsstadien und Krankheitsanfälligkeit her und könnte deshalb ein Ansatzpunkt für Präventionsstrategien sein.

In diesem Projekt sollte ein Mausmodell zur mütterlichen Rauchexposition während der Schwangerschaft etabliert werden, welches dem humanen Phänotyp weitestgehend entspricht.

Erste phänotypische Charakterisierungen der Nachkommen zeigten ein reduziertes Lungen- und Körpergewicht, eine verringerte Lungenfunktion sowie eine gesteigerte Hyperreaktivität der Bronchien. Weiterhin konnte ein vermehrtes Auftreten von glatter Muskulatur (α -SMA) gefunden werden, welches auf Remodellierungsprozesse hindeutet. Analysen der fötalen, pulmonalen Genexpression zeigten eine signifikant verringerte Expression des Wachstumshormones IGF1 der rauchexponierten Nachkommen. Epigenetische Analysen der Histonmodifikationen zeigten einen Trend an verringerter trimethylierter Lysin 27 des Histons H3 (H3K27me3) am *Igf1* Promoter. Diese Histonmodifikation ist bekannt für eine Repression der Translation des zugrunde liegenden Genes. Die damit verbundene Erhöhung der mRNA an *Igf1* konnte in den rauch-exponierten Föten bestätigt werden.

Zusammenfassend lässt sich sagen, dass anhand der Ergebnisse dieses Mausmodells ein direkter Zusammenhang zwischen intrauteriner Zigarettenrauch-Exposition, verringerter Lungenfunktion und dereguliertem Wachstumshormon IGF1 im Jugendlichen zu bestehen scheint. Nach unserem Wissen, ist dieses Mausmodell das erste, welches eine grundlegende Analyse der mütterlichen und fötalen Änderungen nach pränatalem Rauch durchführt.

Die Möglichkeiten unseres Modells erschöpfen sich jedoch darin zu klären, ob die verringerte Lungenentwicklung eine Folge des verringerten Wachstums oder eine direkte Folge des mütterlichen Rauchens ist. Weiterhin kann die Prozedur des Rauchens an sich zu stressbedingten Änderungen in der Entwicklung führen. Zudem bleiben noch eine Reihe offener Fragen: Hat *in utero* Zigarettenrauch auch Einfluss auf die Signaltransduktionswege unterhalb von IGF1 (AKT, STAT, MAPK)? Wird die veränderte Regulation von IGF1 auch an die nächste Generation (F2) ohne zusätzliche Rauchexposition weitergegeben? Sind andere epigenetische Mechanismen, z.B. DNS-Methylierungen, durch Rauchexposition verändert? Weitere Untersuchungen sind nötig, um die zugrunde liegenden Mechanismen der Lungenfunktionsdefizite durch pränatalen Zigarettenrauch zu erklären.

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Chapter 6

Appendix

Table 6.1: Differentially regulated mRNAs in whole lung tissue of intrauterine CS-exposed pups versus control pups on E18.5 evaluated by Affymetrix microarray

Symbol or ID	Gene Description	rawp	FC>1.5x, p<0.05
Spag5	sperm associated antigen 5	0,00033	1,77
Cdc25c	cell division cycle 25C	0,00329	1,76
Bub1b	budding uninhibited by benzimidazoles 1 homolog, beta	0,00101	1,76
Stil	Scf/Tal1 interrupting locus	0,00019	1,75
Cks2	CDC28 protein kinase regulatory subunit 2	0,00150	1,74
LOC100862276	uncharacterized LOC100862276	0,00373	1,71
Ccnb2	cyclin B2	0,00004	1,68
Mis18bp1	MIS18 binding protein 1	0,00200	1,65
Cyt11	cytokine-like 1	0,02300	1,65
Psrc1	proline/serine-rich coiled-coil 1	0,00245	1,64
Crh	corticotropin releasing hormone	0,02131	1,63
1190002F15Rik	RIKEN cDNA 1190002F15 gene	0,00421	1,63
Kif18b	kinesin family member 18B	0,00322	1,63
Kif15	kinesin family member 15	0,00403	1,61
Kif11	kinesin family member 11	0,00180	1,60
Aspm	asp (abnormal spindle)-like, microcephaly associated	0,00123	1,60
Mia1	melanoma inhibitory activity 1	0,02414	1,58
Gm10139	predicted gene 10139	0,02198	1,58
Cdc20b	cell division cycle 20B	0,00614	1,58
Racgap1	Rac GTPase-activating protein 1	0,00057	1,57
Rrm2	ribonucleotide reductase M2	0,00473	1,57
Kif23	kinesin family member 23	0,00512	1,56
Ccna2	cyclin A2	0,00221	1,55
C330027C09Rik	RIKEN cDNA C330027C09 gene	0,00248	1,54
Krtap5-2	keratin associated protein 5-2	0,02530	1,54
Pbk	PDZ binding kinase	0,00443	1,54
Ndc80	NDC80 homolog, kinetochore complex component	0,01081	1,53
Shcbp1	Shc SH2-domain binding protein 1	0,03934	1,52
A630089N07Rik	RIKEN cDNA A630089N07 gene	0,02844	1,52
Cenpk	centromere protein K	0,00787	1,52
Arhgap11a	Rho GTPase activating protein 11A	0,00065	1,52
Ncapd2	non-SMC condensin I complex, subunit D2	0,00320	1,52
Trim59	tripartite motif-containing 59	0,00348	1,51
Nuf2	NUF2, NDC80 kinetochore complex component, homolog	0,00303	1,51

Symbol or ID	Gene Description	rawp	FC > 1.5x, p < 0.05
Bex2	brain expressed X-linked 2	0,00711	-1,51
Fam58b	family with sequence similarity 58, member B	0,00081	-1,51
Gabrp	gamma-aminobutyric acid (GABA) A receptor, pi	0,00282	-1,52
Zfp747	zinc finger protein 747	0,00942	-1,52
My4	myosin, light polypeptide 4	0,02028	-1,52
Mir496	microRNA 496	0,00371	-1,53
Pion	pigeon homolog (Drosophila)	0,00623	-1,53
Ly6c2	lymphocyte antigen 6 complex, locus C2	0,04775	-1,53
Tmem212	transmembrane protein 212	0,00996	-1,53
1810033B17Rik	RIKEN cDNA 1810033B17 gene	0,00344	-1,54
Scgb1a1	secretoglobin, family 1A, member 1 (utero globin)	0,02723	-1,54
Esrra	estrogen related receptor, alpha	0,01113	-1,55
Chad	chondroadherin	0,02077	-1,57
C6	complement component 6	0,02542	-1,58
Cox6a2	cytochrome c oxidase, subunit VI a, polypeptide 2	0,03854	-1,61
Cyp4v3	cytochrome P450, family 4, subfamily v, polypeptide 3	0,00526	-1,62
Car8	carbonic anhydrase 8	0,00282	-1,70
Lyz1	lysozyme 1	0,03971	-1,71
Gm5622	predicted gene 5622	0,00207	-1,71
Scgb3a1	secretoglobin, family 3A, member 1	0,01593	-1,72
Chia	chitinase, acidic	0,00428	-1,72
C3	complement component 3	0,01509	-1,79
Scgb1c1	secretoglobin, family 1C, member 1	0,01313	-1,80
Ear2	eosinophil-associated, ribonuclease A family, member 2	0,00393	-1,81
Chi3l1	chitinase 3-like 1	0,04741	-1,82
Cyp4b1	cytochrome P450, family 4, subfamily b, polypeptide 1	0,00005	-1,83
Car4	carbonic anhydrase 4	0,02578	-1,86
Gdpd2	glycerophosphodiester phosphodiesterase domain containing 2	0,03568	-1,87
Hc	hemolytic complement	0,01395	-1,90
S100g	S100 calcium binding protein G	0,01433	-1,97
Sln	sarcolipin	0,02179	-2,01
Gm15292	predicted gene 15292	0,01104	-2,03
Scd1	stearoyl-Coenzyme A desaturase 1	0,00295	-2,30
Inmt	indolethylamine N-methyltransferase	0,00140	-2,65

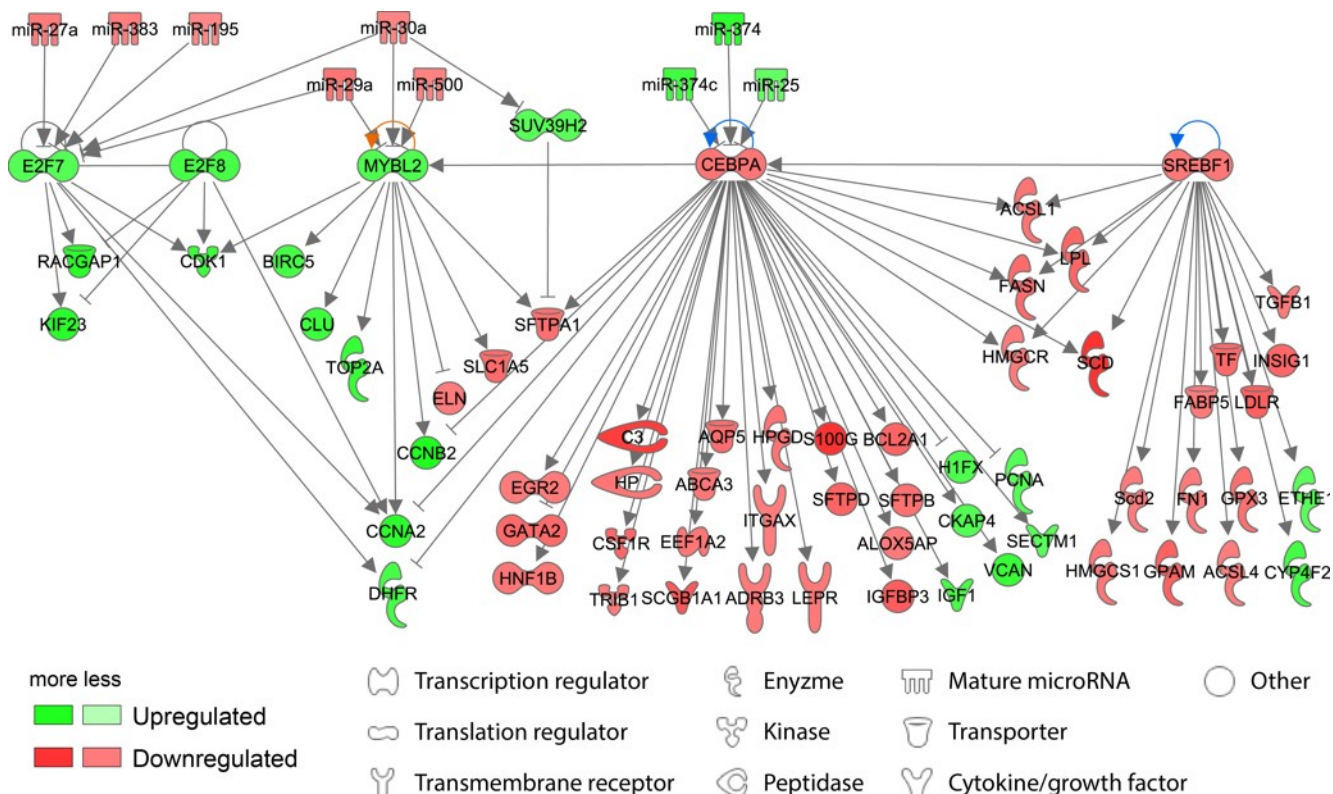


Figure 6.1: Network of dysregulated mRNA and miRNAs after intrauterine CS-exposure on E18.5 Upstream regulators and downstream targets of differentially regulated mRNAs and counter-regulated miRNAs targeting the transcription factors. Network was constructed by Ingenuity pathway analysis. (performed by Stefan Dehmel, CPC Munich)

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1  cactcataag tcttcacgct tcagatactc aacagacgct aggaacgtgt gtctctccct gctgttgca tgctcctttt tgcctttacca gaggtgtgtc taatcaagta
111 catagtaggt gctgtcatca catgattcct tcatcaagtg atacctagca tcacccacca ctggaacagt ctcacaccag gtttctaatag ttcctgtctg aaaggtaaac
221 actgctgtga agtattgacc acacagaggg ttggaaagag cccaagcccc ttgcaaaacc aaactggttt catttgagga caatcaccac atccccaaagc aggtttctat
331 ccatggggca gcgtaaagag gcagtgtaga gtttccaact ggccctctgtc tcaccagatg tgcagatacc ttcaaatccc cctgagagtc cgagagagta agagactgag
441 gcaagtctgg ctcatttcca tctcccctgg gaaagcacac ctggagagat atccgtggaa agcatgcagc gtctaactctg ggcttttcta accttcttc ataattcact
551 ttcttatcta ctgcctctga aagaccactg agaaataggt acaaaccgta tcaacagaag atcagaactc cattccctgt ggcaaaaggcg agtttataca ttataaatag
661 cagaagcagc cggcttgaac catgctgcca gccacttacc cagttgaggg atttgaatga catcataacc ctggagagag tattgttagc cagctgggat tatttgggat
771 acacactcag acacagacac agacagacac acacacttac acacacacac atatgcatgc atgacacat acacacaact gtgtctcaca cacacacaca cacactcatt
881 tatabagaaac aatacagcca acgggaaaca gtgtgtgcct cccatactgc ttccttgggg tcaaggaggt gacaggcatc cagctttgtt gaaaactagg atcattttat
991 ttttcaatcc atcccagatc tcagcatagc ctgattctct gcacaaagca tgacacagtg tctctgacagg agccagccca ctgctgcctg gccatctata ggttttagga
1101 aatgagatca ttccccttgc ctggcaacta ggacaagggg cactctatcc cctccacctc cccttccctc gagagctgcc tttcgatcta gtttaccctg gtcatttcag
1211 ggtaaacagc attgtgcttc ctggagatag tctcttcttc ctttttttct ttttttttct cttcaaatat tgcatattgc ctaaaaatata aagttgcccc cgtgtccacc
1321 ttagatctc taatcttggg gaggtgtatt agcacacaag tgtaccttca aaccctgtgg aaagttaatc agagaacaga gcctattttt ctatggcagc ctcagttatt
1431 aatgtctgct aaccctgtca gagacacaca ttcttttagg gggggggggg gtgagttctc gtgctccagt ttttaagagc gaaggtatga tgttatttgt cacggtgccc
1541 aaaaaagtc ttactogata actttgcccag

```

Figure 6.2: *Igf1* promoter sequence from -1556 to +10 Nucleotide sequence of the 3' end of the murine *igf1* gene (Hall et al 1992). Start of coding region is underlined and dashed lines mark primer pair for qRT-PCR and ChIP products.

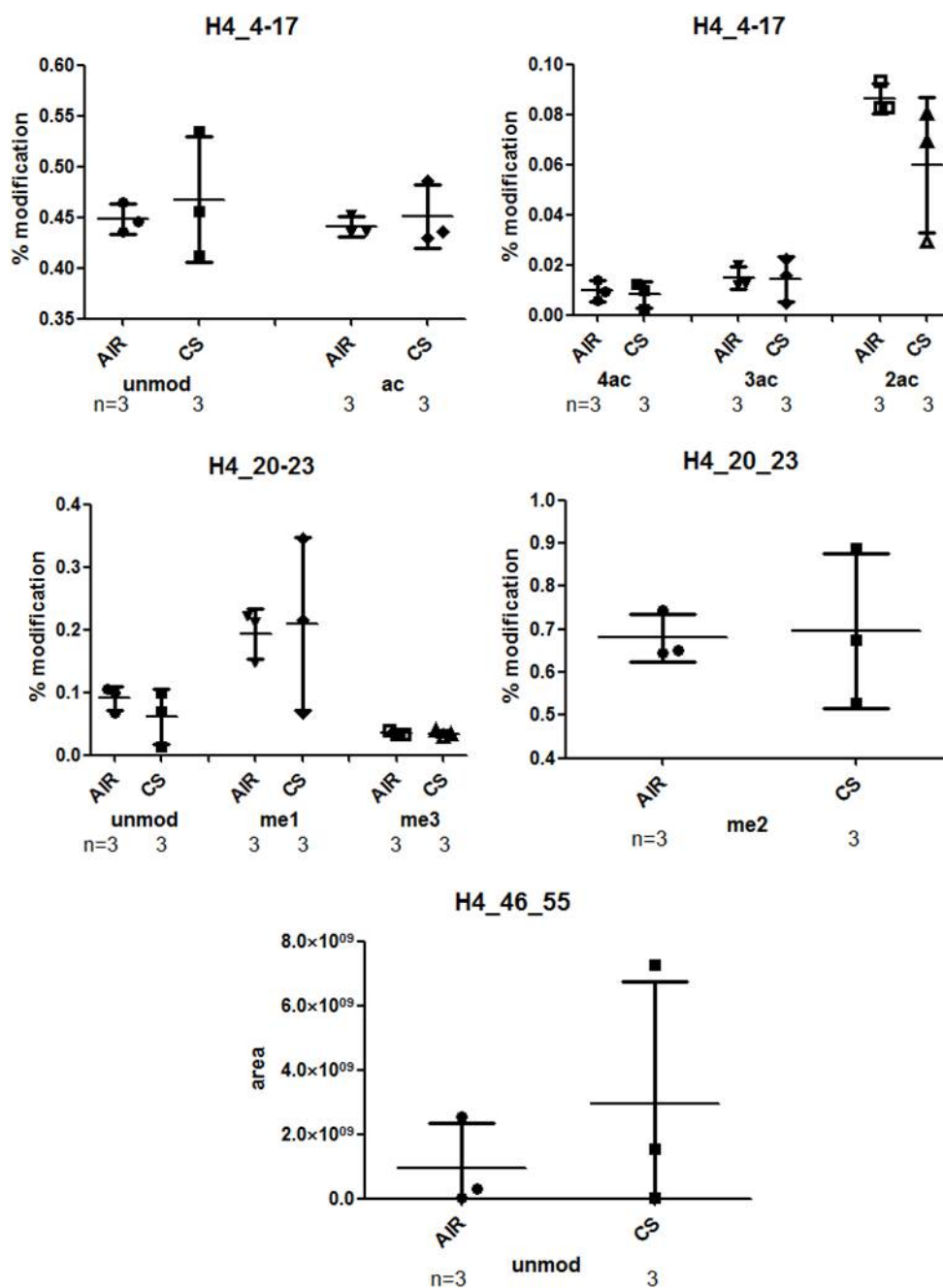


Figure 6.3: **H4 modification pattern after *in utero* CS-exposure**H3 histone of whole lung tissue were isolated. Fragments for mass spectrometry were generated by propionylation (protection of lysin-residues [K]) and further digestion by trypsin, leading to fragments: $H4_4 - 17$, $K5$, $K8$, $K16$ [A]; $H4_{20} - 23$, $K20$ [B]; $H4_{46} - 55$, K . Diamonds(◇) indicate females and males. $n \geq 3$ (≥ 2 batches). Statistical analysis was done by Mann-Whitney-U test

Figure 6.4: *in utero* smoke exposure models Literature summary of intrauterine smoke exposure animal models constructed by Stefan Dehmel

In utero smoke exposure animal models

EXPOSURE	PRECONCEPTUAL	PRENATAL	POSTNATAL (F1/F2/F3)
Environmental tobacco smoke "passive smoking" "sidestream"	- 5,15 - 24,30,42,54,57,58,60 SS 25,31 - 50,52,56,61-63	SS 5,15 SS 24,30,42,54,57,58,60 SS 25,31 SS 50,52,56,61-63	OVA 5,15 SS 24,30,42,54,57,58,60 SS 25,31 - 50,52,56,61-63 SS 43,51/A. fumigatus 43
Maternal smoking "mainstream"	MS 43,51 MS (nose-only) 26 - 27 MS 46 MS 47 MS (nose-only) 49	MS 43,51 MS (nose-only) 26 MS 27 MS 46 MS 47 MS (nose-only) 49	HDM 26 - 27 - 46 MS 47 MS (nose-only) 49
"Active smoking" MS+SS	- 53	MS+SS vs SS 53	- 53
Surrogate	- 28,44,45 - 59 nicotine 55 nicotine (F1) 29,64	nicotine 28,44,45,48 nicotine 59 nicotine 55 - F2 ²⁹ , F3 ⁶⁴	nicotine 28,44,45,48 - 59 nicotine 55 - F2 ²⁹ , F3 ⁶⁴

Mainstream studies

- ²⁶ Blacquièrre, Hylkema, *et al.*, 2009, Penh
²⁷ Larcombe, Sly, *et al.*, 2011, wave-tube system
⁴³ Singh, Sopori, *et al.*, 2003, Penh
⁴⁶ Haley, Tantisara, *et al.*, 2011, hum.+mur., no AHR, Runx1 SNPs
⁴⁷ Manoli, Haley, *et al.*, 2012, no AHR m.
⁴⁹ Gaworski, Rajendran, *et al.*, 2004, rats, neuro study

Sidestream studies

- ⁵ Penn, Lomax, *et al.*, 2007, Penh
¹⁵ Rouse, Penn, *et al.*, 2007, Penh referenced
²⁴ Wu, Dey, *et al.*, 2009, AHR m.
²⁵ Singh, Sopori, *et al.*, 2011, AHR m.

Sidestream studies, cont.

- ³⁰ Wang, Pinkerton, *et al.*, 2008, primates, Th1 cyto., no AHR m.
³¹ Singh, Sopori, *et al.*, 2009, METH only 2 genes
⁴² Xiao, Penn, *et al.*, 2012, Penh
⁴³ Singh, Sopori, *et al.*, 2003, Penh
⁵⁰ Yang, Penn, Ballinger, *et al.*, 2004, atherogenesis study, no AHR m.
⁵¹ Singh, Sopori, *et al.*, 2006, splenic T cell responses, no AHR m.
⁵² Gandley, DeLoia, *et al.*, 2010, vascular study, no AHR m.
⁵⁴ Joad, Pinkerton, *et al.*, 1995, rats, isolated perf. lungs for AHR m.
⁵⁶ Mukhopadhyay, Pisano, *et al.*, hippocamp. gene expr. study, no AHR m.
⁵⁷ Westbrook, Ballinger, *et al.*, vasc. ox. stress study, primates, no AHR m.
⁵⁸ Rajini, Witschi, *et al.*, 1994, fetal growth study, rats, no AHR m.
⁶⁰ Gospe, Pinkerton, *et al.*, 1996, rats, brain dev. study, no AHR m.
⁶¹ Ji, Pinkerton, *et al.*, 1999, rats, CC10 fetal expr., no AHR m.
⁶² Joad, Pinkerton, *et al.*, 1998, rats, isolated perf. lungs for AHR m.
⁶³ Lee, Pinkerton, *et al.*, 2000, rats, P450 study, no AHR m.

Active smoking (MS+SS)

- ⁵³ Esposito, Pisano, *et al.*, 2008, fetal growth study, no AHR

Surrogate studies

- ²⁸ Wongtrakool, Spindel, *et al.*, 2012, AHR m.
²⁹ Rehan, Torday, *et al.*, 2012, rats, AHR m., global DNA meth & H ace in F2
⁴⁴ Liu, Rehan, *et al.*, 2011, rats, AHR m.
⁴⁵ Liu, Rehan, *et al.*, 2012, rats, AHR m.
⁴⁸ Wongtrakool, Roman, *et al.*, 2007, nicotine exposed murine fetal lung cultures
⁵⁵ Wongtrakool, Gauthier, *et al.*, 2012, no AHR
⁵⁹ Witschi, Last, *et al.*, 1994, pregnancy outcomes, no AHR
⁶⁴ Rehan, Torday, *et al.*, 2013, in rats, AHR m.

Human patient cohort studies

- Breton, Gilliland, *et al.*, 2009, METH 1031 CpG loci

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02/2015	Research fellowship in Groningen (NL); Group: Pathology & Medical Biology Dr. M. Hylkema
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09/2001 – 07/2002	Study of Bioinformatics at FH Weihenstephan in Freising
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Eidesstattliche Versicherung

Hiermit erkläre ich, Petra Nathan, an Eides statt, dass ich die hier vorliegende Dissertation mit dem Titel

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Ort, Datum

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