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THE EFFECTS OF MATERNAL HYPERGLYCEMIA ON HUMAN UMBILICAL VASCULAR GENE EXPRESSION AND NEONATAL RAT LUNG DEVELOPMENT

by

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To my family

ABSTRACT

Anna Koskinen: The effects of maternal hyperglycemia on human umbilical vascular gene expression and neonatal rat lung development.

Department of Pediatrics, Research Centre of Applied and Preventive Cardiovascular Medicine (CAPC), University of Turku, Turku, Finland. Annales Universitatis Turkuensis, Medica-Odontologica, 2014

Background: Maternal diabetes affects many fetal organ systems, including the vasculature and the lungs. The offspring of diabetic mothers have respiratory adaptation problems after birth. The mechanisms are multifactorial and the effects are prolonged during the postnatal period. An increasing incidence of diabetic pregnancies accentuates the importance of identifying the pathological mechanisms, which cause the metabolic and genetic changes that occur in offspring, born to diabetic mothers.

Aims and methods: The aim of this thesis was to determine changes both in human umbilical cord exposed to maternal type 1 diabetes and in neonatal rat lungs after streptozotocin-induced maternal hyperglycemia, during pregnancy. Rat lungs were used as a model for the potential disease mechanisms. Gene expression alterations were determined in human umbilical cords at birth and in rat pup lungs at two week of age. During the first two postnatal weeks, rat lung development was studied morphologically and histologically. Further, the effect of postnatal hyperoxia on hyperglycemia-primed rat lungs was investigated at one week of age to mimic the clinical situation of supplemental oxygen treatment.

Results: In the umbilical cord, maternal diabetes had a major negative effect on the expression of genes involved in blood vessel development. The genes regulating vascular tone were also affected. In neonatal rat lungs, intrauterine hyperglycemia had a prolonged effect on gene expression during late alveolarization. The most affected pathway was the upregulation of extracellular matrix proteins. Newborn rat lungs exposed to intrauterine hyperglycemia had thinner saccular walls without changes in airspace size, a smaller relative lung weight and lung total tissue area, and increased cellular apoptosis and proliferation compared to control lungs, possibly reflecting an aberrant maturational adaptation. At one and two weeks of age, cell proliferation and secondary crest formation were accelerated in hyperglycemia-exposed lungs. Postnatal hyperoxic exposure, alone caused arrested alveolarization with thin-walled and enlarged alveoli. In contrast, the dual exposure of intrauterine hyperglycemia and postnatal hyperoxia resulted in the phenotype of thick septa together with arrested alveolarization and decreased number of small pulmonary arteries.

Conclusions: Maternal diabetic environment seems to alter the umbilical cord gene expression profile of the regulation of vascular development and function. Fetal hyperglycemia may additionally affect the genetic regulation of the postnatal lung development and may actually induce prolonged structural alterations in neonatal lungs together with a modifying effect on the deleterious pulmonary exposure of postnatal hyperoxia. This, combined with the novel human umbilical cord gene data could serve as stepping stones for future therapies to curb developmental aberrations.

Key words: diabetes, hyperglycemia, umbilical cord, neonatal lung, lung morphology, microarray analysis, apoptosis, proliferation, hyperoxia

LYHENNELMÄ

Anna Koskinen: Äidin hyperglykemian vaikutus geenien ilmentymiseen napanuorassa ja syntymän jälkeiseen rotan keuhkojen kehitykseen

Lastentautioppi, Sydäntutkimuskeskus, Turun yliopisto, Turku. Annales Universitatis Turkuensis, Medica-Odontologica, 2014

Tausta: Äidin diabetes vaikuttaa kehittyvään sikiöön aiheuttaen monenlaisia lyhyt- tai pitkäkestoisia ongelmia syntymän jälkeen, kuten keuhkojen toiminnan tai verenkierron häiriöitä. Diabeteksen ja diabeetikkoraskauksien yleistyessä on tärkeää oppia tuntemaan niitä mekanismeja, jotka johtavat sikiön poikkeavaan kehitykseen tai ohjelmoitumiseen.

Tavoitteet ja menetelmät: Tässä väitöskirjatyössä selvitettiin sekä äidin tyypin 1 diabeteksen vaikutusta napanuoran geenien ilmentymiseen että kokeellisen streptosotosiinilla aiheutetun hyperglykemian vaikutusta rotanpoikasten keuhkojen kehitykseen. Rottamallia käytettiin siis mallintamaan äidin diabeteksen vaikutuksia kehittyvissä keuhkoissa. Ihmisnapanuoran lisäksi geenien ilmentymisprofiili tutkittiin rotanpoikasten keuhkoista kahden viikon iässä eli myöhäisessä alveolarisaatiovaiheessa. Lisäksi rottien keuhkojen kehitystä tarkasteltiin morfologisesti ja histologisesti syntymästä kahden viikon ikään asti. Hoidollista tilannetta mallinnettiin altistamalla emon hyperglykemialle altistuneet keuhkot vielä syntymänjälkeiselle lisähapelle. Näiden keuhkojen kehitystä tarkasteltiin viikon ikäisillä poikasilla.

Tulokset: Äidin diabetes vähensi verisuonten kehitystä säätelevien geenien ilmentymistä napanuorassa. Lisäksi verisuonten toimintaa säätelevien geenien ilmentyminen muuttui. Sikiöaikainen altistuminen hyperglykemialle aiheutti rotanpoikasten keuhkoissa muutoksia geenien ilmentymisessä vielä kahden viikon iässä, erityisesti solunulkoisen tukikudoksen rakennetta säätelevissä geeneissä. Hyperglykemia-altistus ohensi vastasyntyneen rotan keuhkorakkuloiden seinämiä vaikuttamatta kuitenkaan ilmatilojen kokoon, pienensi keuhkojen suhteellista painoa ja kudospinta-alaa, ja lisäsi solukuolemaa ja toisaalta solujen jakaantumista keuhkokudoksessa verrattuna terveen emon poikasten keuhkoihin. Viikon ja kahden viikon iässä solujen jakaantuminen ja sekundaaristen seinämien muodostus keuhkorakkuloissa olivat edelleen kiihtyneet. Syntymänjälkeinen lisähappialtistus yksinään aiheutti tyypillisen keuhkovaurion ohutseinäisine laajentuneine keuhkorakkuloineen, mutta kaksoisaltistuksessa eli emon hyperglykemialle jo altistuneissa keuhkoissa se aiheutti alveolarisaatiohäiriön, johon liittyi paksuksi jääneet keuhkorakkuloiden seinämät. Näissä seinämissä näkyi vähemmän pieniä keuhkoverisuonia kuin kontrollikeuhkoissa.

Päätelmät: Äidin diabeteksen vaikutus näkyy napanuorassa verisuonten kehitystä ja toimintaa säätelevien geenien poikkeavassa ilmentymisessä. Kehittyvissä keuhkoissa sikiöaikainen hyperglykemialle altistuminen saattaa muuttaa geenien ilmentymistä pitkäaikaisesti syntymän jälkeen ja aiheuttaa keuhkoihin rakenteellisia muutoksia, sekä muuntaa syntymänjälkeisen lisähapen aiheuttamaa keuhkovauriota. Nämä keuhkolöydökset yhdessä ihmisnapanuorista saadun geenidatan kanssa ovat uutta tietoa, joka voidaan ottaa huomioon suunnitellessa tulevaisuuden hoitoja äidin diabeteksen aiheuttamiin kehitysongelmiin.

Avainsanat: diabetes, hyperglykemia, napanuora, vastasyntyneen keuhkot, keuhkojen morfologia, mikrosiruanalyysi, solukuolema, solujen jakaantuminen, hyperoksia

TABLE OF CONTENTS

ABBREVIATIONS

ASMA	*Alpha*-Smooth Muscle Actin
BMP4	Bone morphogenetic protein 4
CD45, LCA	Leukocyte common antigen
CT	Computed tomography
Cthrc1	Collagen triple helix repeat containing 1
Col1a1	Collagen type I *alpha* 1
Col3a1	Collagen type III *alpha* 1
COL8A1	Collagen, type VIII, *alpha* 1
DAVID	Database for Annotation, Visualization, Integrated Discovery
DLL1	*Delta*-like 1
DM pups	Pups of diabetic dams
EDN1	Endothelin 1
EDNRB	Endothelin receptor type B
FC	Fold change
GHbA1c	Blood glycosylated hemoglobin
GO	Gene ontology
Gpx3	Glutathione peroxidase 3
GSH	Glutathione reduced form
GSSG	Glutathione oxidized form
HE	Hematoxylin-eosin
Igfbp2	Insulin-like growth factor binding protein 2
MEF2C	Myocyte enhancer factor 2C
Mmp14	Matrix metalloproteinase 14
MMP2	Matrix metalloproteinase 2
NPPB	Natriuretic peptide precursor B
PCA	Principal Component Analysis
PCNA	Proliferating cell nuclear antigen
PDE5A	Phosphodiesterase 5A
Pdgfra	Platelet derived growth factor receptor *alpha*
PTGS1, COX1	Prostaglandin-endoperoxide synthase 1, cyclooxygenase 1
RT-PCR	Real-time Polymerase Chain Reaction
Sod3, EC-SOD	Superoxide dismutase 3
STZ	Streptozotocin
TUNEL	Terminal transferase-mediated dUTP nick end-labelling

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I - IV.

I Koskinen A, Lehtoranta L, Laiho A, Laine J, Kääpä P, Soukka H. Maternal diabetes-induced gene expression changes in the umbilical cord. Submitted.

II Koskinen A, Laiho A, Lukkarinen H, Kääpä P, Soukka H. Maternal hyperglycemia modifies extracellular matrix signaling pathways in neonatal rat lung. Neonatology 2010; 98(4):387-396.

III Koskinen A, Lukkarinen H, Moritz N, Aho H, Kääpä P, Soukka H. Fetal hyperglycemia alters lung structural development in neonatal rat. Pediatr Pulmonol 2012; 47(3):275-82.

IV Koskinen A, Lukkarinen H, Laine J, Ahotupa M, Kääpä P, Soukka H. Delay in rat lung alveolarization after the combined exposure of maternal hyperglycemia and postnatal hyperoxia. Pediatr Pulmonol 2013; Jul 8. doi: 10.1002/ppul.22837.

1 INTRODUCTION

Maternal diabetes may interfere with the development of almost every organ in the fetus (Weindling, 2009). Despite overall good glycemic control during pregnancy, the risk for an adverse pregnancy outcome remains increased in type 1 diabetic pregnancies (Evers et al., 2004; McCance, 2011; Murphy et al., 2011). Most of the fetal and neonatal consequences are linked to transplacental transmission of glucose with resultant fetal hyperinsulinemia and β-cell hyperplasia (Pedersen, 1971). The underlying biological mechanisms of maternal hyperglycemia are suggested to be multifactorial. The diabetic exposure *in utero* may indeed have a direct effect on the fetal vasculature, and may predispose the offspring to metabolic derangements or can also induce genetic or epigenetic changes (El-Osta et al., 2008; Marco et al., 2012).

The umbilical cord is a fetus-derived organ. It is used to assess the biological responses to different pregnancy-associated conditions. The umbilical cord cells possess a wide differentiation potential and plasticity, when subjected to many pregnancy-induced changes (Can and Karahuseyinoglu, 2007). In diabetic pregnancy, umbilical cords show structural abnormalities in different tissue compartments (Asmussen, 1980; Cromi et al., 2007; Singh, 1986). Recently, however, the whole umbilical cord tissue was successfully used for genetic studies in pregnancies complicated with intrauterine growth restriction (Hussain et al., 2008; Lim et al., 2012).

In the perinatal lungs, maternal hyperglycemia may induce metabolic derangement leading to pulmonary biochemical and physiological immaturity (Gewolb et al., 1985; Thulesen et al., 2000). Indeed, infant wellbeing of diabetic mothers is often at risk for respiratory distress associated with surfactant deficiency (Langer, 2002) and delay in the immediate postnatal cardiopulmonary adaptation (Seppänen et al., 1997; Vela-Huerta et al., 2007). Previous experimental data indicate that maternal diabetes may be also associated with fetal lung morphological changes, such as a thickened septa, reduced air-mesenchyme ratio and lower distal airspace size (Gewolb et al., 1985; Pinter et al., 1991; Thulesen et al., 2000), as well as delayed development of endothelial and type II epithelial cell basement membrane and decreased expansion of pulmonary capillary network (Grant et al., 1984). These alterations are not completely reversed during restoration of normoglycemia after birth. This suggests that prolonged intrauterine hyperglycemic exposure affects alveolarization and microvascular maturation (Grant et al., 1984). Further, little information exists about the priming effect of fetal hyperglycemic exposure on postnatal lung reactivity, although increased oxygen tolerance was reported in neonatal rat lungs of hyperglycemic dams (Sosenko and Frank, 1986).

In an era of increasing incidence of type 1 diabetes and diabetic pregnancies (Lammi et al., 2008; Maahs et al., 2010), expanding the knowledge about maternal diabetes-related perinatal alterations is of utmost importance. The main objective of this thesis

was to investigate the effects of experimental maternal diabetes, in a rat model, on neonatal lung gene expression and the structural development and the combined effect of postnatal hyperoxia on maternal hyperglycemia-primed lungs. These studies were complemented with *in vitro* investigations into the effect of maternal type 1 diabetes on human umbilical cord tissue gene expression profiles.

2 REVIEW OF THE LITERATURE

2.1 The infant of a diabetic mother

Maternal diabetes associates with a wide range of adverse effects on fetal and neonatal organ systems, including the lungs (Nold and Georgieff, 2004). Although advances in maternal and fetal care for diabetes improve the prognosis of the newborns, they more frequently require intensive care than the offspring of non-diabetic mothers (Cordero et al., 1998; Menacker and Martin, 2008). Simultaneously, the incidence of type 1 diabetes is increasing worldwide, with approximately a 4 percent increase per year in Finnish children and young adults (Lammi et al., 2008; Maahs et al., 2010). In addition, among the youth, the incidence of type 2 diabetes is markedly increasing (Badaru and Pihoker, 2012; Lammi et al., 2008). Thus, the incidence of infants, from diabetic mothers, is rising.

Infants of diabetic mothers are at risk for preterm birth, macrosomia, asphyxia, respiratory distress, hypoglycemia, hypocalsemia, hyperbilirubinemia, polycytemia and hyperviscosity, hypertrophic cardiomyopathy, and congenital anomalies (Cordero et al., 1998; Eidem et al., 2010; Hay, 2012; Kitzmiller et al., 1996; Weindling, 2009). Additionally, infants of diabetic mothers may have an increased risk for long-term adverse neurodevelopmental sequelae (Hawdon, 2011). Most of the fetal and neonatal complications are related to altered maternal glycemic control (Hay, 2012; Nold and Georgieff, 2004). Maternal hyperglycemia results in fetal hyperglycemia because glucose traverses the placental membrane, while insulin is unable to cross the placenta (Pedersen, 1971). Thus, the main pathologic condition to which the embryo or fetus is subjected during early pregnancy is hyperglycemia. After 20 weeks gestation, the fetal pancreas is capable of its own insulin secretion. The fetus responds to hyperglycemia with hypertrophy of fetal pancreatic islets and hyperinsulinemia, further affecting the wellbeing of the fetus and newborn during late pregnancy (Nold and Georgieff, 2004) (Figure 1).

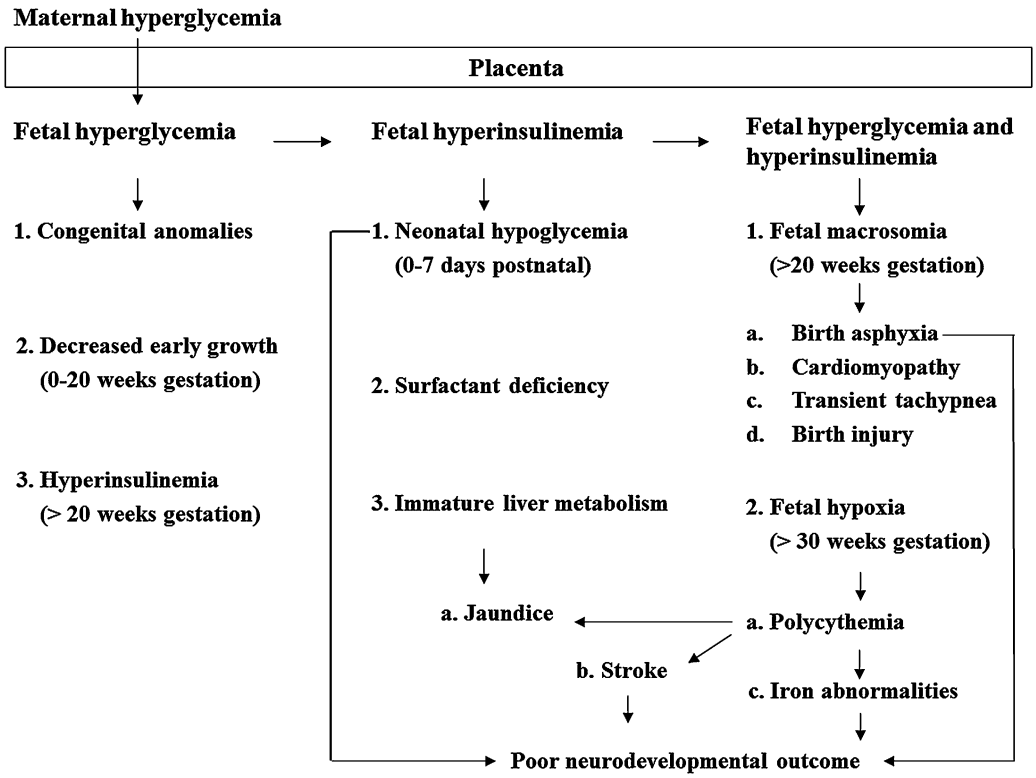

Figure 1. The effect of fetal hyperglycemia or fetal hyperinsulinemia, or both in synergy, on the fetus and neonate (figure reprinted and adapted from an article by Nold and Georgieff, 2004 with permission from Elsevier Limited, Oxford, UK).

2.1.1 Pathophysiological alterations in infants of diabetic mothers

During organogenesis, diabetic embryopathy-associated birth defects are induced in the first gestational weeks. The most common malformations develop in the cardiovascular system, central nervous system or renal and urinary system. Spine developmental defects, especially caudal dysgenesis, or limb and rib deficiencies also occur (Correa et al., 2008; Martínez-Frías, 1994). Hyperglycemia affecting the embryo is mainly responsible for these malformations (Reece, 2012), but other teratogenic factors, secondary to poor hyperglycemic control, may be involved. Aberrant gene signalling pathways controlling apoptosis and proliferation as well as deficient antioxidative defense in the pathophysiology of experimental diabetic embryopathy may also be disease manifestations (Moley, 2001; Ornoy, 2007; Reece, 2012; Zabihi and Loeken, 2010).

Excess glucose metabolism, in the developing embryo, may alter the metabolism of arachidonic acid and decrease the production of prostaglandins, overactivate the polyol pathway, alter glycation of proteins, decrease the production of the cell membrane component myo-inositol, and increase the intracellular level of free oxygen radicals leading to diabetic embryopathy (Eriksson, 2009; Reece, 2012). Further, excess glucose delivery into fetuses induces oxidative stress via increased

mitochondrial respiration (i.e., increased glucose oxidation) and oxygen utilization leading to hypoxic stress, increased mitochondrial superoxide production and decreased availability of cellular antioxidant glutathione (GSH) (Li et al., 2005; Ornoy, 2007; Zabihi and Loeken, 2010).

During late pregnancy, chronic fetal hypoxia is more frequent in diabetic pregnancies due to fetal macrosomia and increased oxygen consumption together with placental dysfunction (Nold and Georgieff, 2004; Schwartz and Teramo, 2000). Maternal hyperglycemia-induced placental oxidative stress may further increase embryonic and fetal oxidative strain (Ornoy, 2007). Indeed, newborns of diabetic mothers have increased oxidative species in their cord blood in relation to the degree of maternal hyperglycemia (Sarikabadayi et al., 2011).

Maternal diabetes may cause phenotypic modifications in fetal tissues producing long-term functional and structural changes (Gluckman et al., 2008; Simeoni and Barker, 2009). The underlying biological mechanisms are multifactorial and metabolic derangements, changes in gene expression and epigenetics may be causes (Marco et al., 2012). Indeed, circulating endothelial progenitor cells in human fetal umbilical cords of term diabetic pregnancies show reduced functions of colony formation, self-renewal capacity and capillary-like tube formation, which are critical components of vasculogenesis (Ingram et al., 2008). Also, the umbilical cord blood of newborns from type 1 diabetic pregnancies show a decreased level of circulating vascular endothelial growth factor and increased levels of troponin T, pro-B-type natriuretic peptide and pro-A-type natriuretic peptide - markers of cardiac dysfunction (Lassus et al., 2003; Russell et al., 2009; Smith et al., 2013). *In vitro* studies demonstrate that even transient exposure of human aortic endothelial cells to hyperglycemia induced epigenetic changes that cause persistent expression of proatherogenic genes (MCP1 and VCAM1) despite a subsequent restoration to normoglycemia (El-Osta et al., 2008). Similarly, children of type 1 or gestational diabetic mothers have increased levels of circulating endothelial cell adhesion molecules, such as E-selectin and vascular adhesion molecule 1 and these are predictors of endothelial failure and the earliest preclinical stage of cardiovascular disease (Manderson et al., 2002; West et al., 2011). These infants are more obese and have higher systolic and mean arterial blood pressure as well as a higher 2-hour glucose and insulin values at the age of 10-16 years than newborns of non-diabetic mothers (Cho et al., 2000). Young adult offspring of type 1 or gestational diabetic mothers have additionally two-fold risk of obesity, a 2.5 to 4-fold risk of metabolic syndrome, and a 4 to 7- fold risk of type 2 diabetes, which associates with maternal blood glucose level in late pregnancy (Clausen et al., 2008). This increased risk is likely to be due to the exposure to maternal hyperglycemia and not lifestyle or genetic factors (Clausen et al., 2009).

2.1.2 Experimental diabetic embryopathy

Animal studies have provided most of the current information about the effects of maternal hyperglycemia on fetal development. The discovery of streptozotocin (STZ), which chemically destroys pancreatic β-cells in laboratory animals, has enabled studies on fetal events in diabetic pregnancy (Junod et al., 1969).

Hyperglycemia induces embryonal vasculopathy. Murine embryos exposed to maternal STZ-induced hyperglycemia or *in vitro* hyperglycemia have arrested primary capillary plexus formation, defects in umbilical vessel formation, and decreased expression and protein level of vascular endothelial growth factor A with abnormal signalling of its receptor (Pinter et al., 2001; Pinter et al., 1999). Further, chicken embryos exposed to hyperglycemia show impaired angiogenesis through the induction of apoptosis and decreased proliferation of endothelial cells but without the effect on vascular growth factor expression (Larger et al., 2004). Fetal rats and rabbits exposed to maternal hyperglycemia lack the proper migration of pulmonary capillaries and fusion of their basement membrane (Grant et al., 1984; Sosenko et al., 1980). During adulthood, rats exposed to intrauterine STZ-induced hyperglycemia show hypertension, baroreflex dysfunction, renin-angiotensin system activation, reduce the response to endothelium-dependent vasodilators and enhanced norepinephrine-induced vasoconstriction as signs of vascular dysfunction (Holemans et al., 1999; Wichi et al., 2005). Further, maternal hyperglycemia-exposed fetal rats show cardiac hyperplasia and dysfunction (Lehtoranta et al., 2013), as well as local accumulation of glycation products and decreased expression of vascular endothelial growth factor in the regions that are most sensitive to diabetes-induced congenital heart defects (Roest et al., 2009).

Gene expression profile analyses exist, which assess the differences in multiple genes and pathways in rat and mouse embryos in response to a diabetic environment. In these studies, hyperglycemia induces cessation of cell proliferation in murine tissues via upregulation of growth inhibitors and downregulation of cell division stimulators (Reece et al., 2006). It also affect expression of murine genes involved in oxidative stress, hypoxia (Pavlinkova et al., 2009), apoptosis, proliferation, migration, and differentiation (Jiang et al., 2008; Vijaya et al., 2013). Similarly, mice neural stem cells exposed to high glucose concentration have gene expression profiles representing decreased cell proliferation, increased apoptosis, and altered cell-cycle progression and cell-fate specification during neurulation (Fu et al., 2006).

A few sporadic genes may be involved in controlling apoptosis in malformed rat embryos exposed to maternal hyperglycemia: the proapoptotic BAX gene; antiapoptotic BCL2 and Akt genes (Gäreskog et al., 2007; Reece et al., 2005); antioxidative copper zinc and manganese superoxide dismutases and glutathione peroxidase 1 (Wentzel et al., 2008); neural tube and cardiac outflow tract developmental gene Pax3 (Kumar et al., 2007; Morgan et al., 2008; Phelan et al., 1997); vascular endothelial growth factor A (Wentzel et al., 2008) and bone morphogenetic protein 4 (Kumar et al., 2007; Wentzel et al., 2008). In fetal rat kidneys, maternal hyperglycemia causes the downregulation of matrix metalloproteinases, simultaneously with altered morphology (Duong Van Huyen et al., 2007).

2.2 Umbilical cord and diabetic pregnancy

Umbilical cord is a fetus-derived organ that connects the fetus to the placenta. It is made of two arteries and a vein surrounded by a soft connective tissue called Wharton´s

jelly, which is composed of mesenchymal, myofibroblast-type stromal cells, occasional mast cells and abundant extracellular matrix rich in water, collagens and proteoglycans, mainly hyaluronic acid. The uppermost covering of the umbilical cord is from amniotic epithelium (Can and Karahuseyinoglu, 2007; Nanaev et al., 1997). Stromal cells are multipotent cells having certain plasticity and developmental flexibility and are considered as a source of stem cells. The connective tissue has a major role in preventing the vessels from bending and occluding and the stromal cells are suggested to participate in regulation of umbilical cord blood flow (Can and Karahuseyinoglu, 2007). The positioning of placental fetal vasculature exposes the fetal vessels directly to maternal metabolic milieu and along the umbilical cord, the vasculature is continuous with the fetal circulation. In the offspring, fetal-maternal interactions in diabetic pregnancy may result in metabolic disturbances leading to fetoplacental endothelial (Westermeier et al., 2009) and further vascular dysfunction (Leiva et al., 2011).

2.2.1 Genetic changes in the umbilical cord from diabetic pregnancy

Studies concerning the effects of maternal diabetes on umbilical cord gene expression are still very limited. A marker of endothelial dysfunction, intercellular adhesion molecule-1, has no difference in protein expression level in umbilical and placental vascular tissue of gestational diabetic pregnancies compared to non-diabetic controls (Kurt et al., 2010). However, cultured endothelial cells from human umbilical vein in gestational diabetic pregnancy show increased expression and activity of endothelial nitric oxide synthase, indicating altered vascular reactivity (Sobrevia et al., 2011). In the placenta, gestational diabetes causes altered expression of immune-related genes (Zhao et al., 2011), increases the gene expression of antioxidants (Lappas et al., 2010) and affects the levels or protein and gene expressions of vascular endothelial growth factors (Leach, 2011; Marini et al., 2008).

2.2.2 Morphological changes in umbilical cords from diabetic pregnancies

Maternal diabetes induces alterations in the ultrastructure of the umbilical cord of newborn infants. These changes include defects in extracellular matrix and vessel wall modeling. A rupture of the collagen fibers and oedema (Cromi et al., 2007; Singh, 1986), basement membrane arterial thickening and intimal cushions with glycogen accumulation both in the cells of intima and media (Asmussen, 1980), rupture of the endothelial lining of the arteries, an unduly dilated vein, and the disruption of smooth muscle fibers surrounding the vessels (Singh, 1986) are all the observed defects. The proportion of Wharton´s jelly area detected by obstetric ultrasound is increased in fetal umbilical cords in gestational diabetic pregnancies (Cromi et al., 2007) and among the infants of gestational diabetic mothers, macrosomia appears to increase the thickness of umbilical artery intima-media layer (Sarikabadayi et al., 2012).

In placenta, maternal type 1 diabetes is associated with enhanced angiogenesis with increased surface area of the villous fetal capillary wall (Jauniaux and Burton, 2006; Jirkovská et al., 2012; Leach, 2011). Capillaries have either smaller or larger diameter

but they are more branched (Jirkovská et al., 2012). Enhanced fetoplacental angiogenesis in type 1 diabetic pregnancies occurs mainly by longitunal growth, i.e., the combined length of capillaries is increased (Mayhew, 2002). The endothelial cells of the fetal villous capillaries are immature and enlarged with the subsequent reduction in the size of the capillary lumen in diabetic pregnancies (Jones and Fox, 1976). In type 1 diabetes, villous capillaries show a loss of junctional adhesion molecules (Leach, 2011).

2.3 Neonatal lungs and maternal hyperglycemia

2.3.1 Normal lung development

Lung development can be divided into five distinct stages of morphogenesis: embryonic; pseudoglandular; canalicular; saccular; and alveolar stage. Branching morphogenesis, development of the air-conducting system, takes place during the first two stages. Expansion and maturation of the lungs happens during the latter three stages. In humans, the saccular stage occurs between the 24th and 38th week of pregnancy and the alveolarization begins around the 36th - 38th week of pregnancy. The major phase of alveolarization occurs postnatally, with more than 90 percent of all alveoli being formed after birth, and lasting over at least the first 6 months of life. The rat lung is more immature at birth than human lung. In rats, mature pups, born at day 22 of gestation, are in the saccular stage of lung development and the lungs undergo an intensive period of alveolarization between the 4th to 14th days of life. Roughly, the postnatal developmental steps of rat lungs resemble that in human lungs (Burri, 2006; Pringle, 1986).

Alveoli are formed by the septation of large saccules that constitute the gas-exchange region of the immature lung (Bourbon et al., 2009)(Figure 2). At birth, immature airspace walls, i.e., primary septa consists of two capillary layers with central sheet of connective tissue (Burri, 2006). Shortly after birth, a secondary septa, i.e., alveolar septa emerge from the primary septa by the folding up of one of the two capillary layers, thereby increasing lung surface area (Burri, 2006). This process of septation appears to be driven by the smooth muscle cells, elastin and collagen that make up the secondary crests and attract into them the capillary from the primary septa (Galambos and Demello, 2008). The septation is driven by transcription factors and growth factors that affect extracellular matrix molecules (Galambos and Demello, 2008). At the initiation of septation, fibroblasts proliferate and produce abundant elastin at the origin of the secondary crests to cause the secondary septa to grow. Fibroblast proliferation then decreases in the proximal septa but continues at the distal septal tips during alveolarization (McGowan and Torday, 1997). Type 1 epithelial cells differentiate to line the surface area of the distal lung and their basement membrane fuses with the pulmonary capillary basement membrane such that gas-exchange surface develops. Type 2 epithelial cells synthesize and secrete increasing amounts of surfactant, serve as progenitor cell for type 1 cells, and are involved in innate host defense (Shannon and Hyatt, 2004). Thinning of alveolar walls occurs through programmed cell death of interstitial fibroblasts and type 2 epithelial cells (De Paepe

et al., 1999; Kresch et al., 1998; Massaro and Massaro, 2002; Schittny et al., 1998). Lipofibroblasts are present in the alveolar interstitium, synthesize collagen and elastin, and assist the type 2 cells in the synthesis of surfactant (McGowan and Torday, 1997). They become highly apoptotic over the first 1–2 week of postnatal life, highlighting the importance of their removal process during alveolar thinning (Awonusonu et al., 1999).

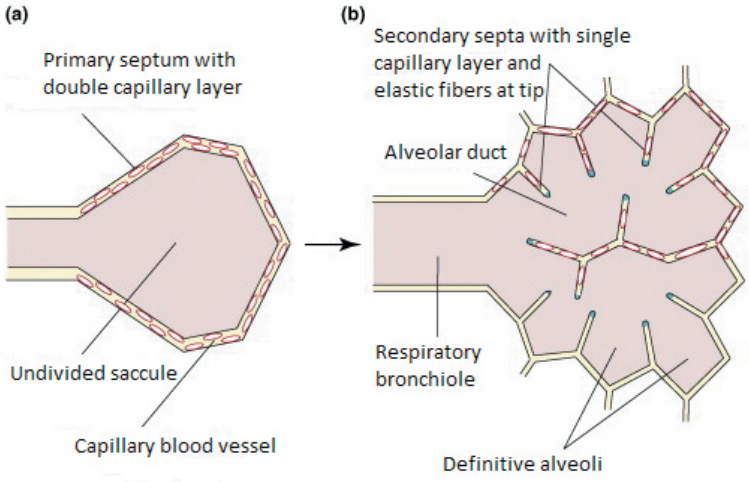

Figure 2: Schematic representation of the alveolarization in normal lung development. The saccular air space (a) develops into the alveolar structure (b) by the growth of secondary septa and the fusion of the capillary vessels into single central layers (figure reprinted and adapted from an article by Bourbon et al., 2009 with permission from Elsevier Limited, Oxford, UK).

During and after alveolarization occurs the microvascular maturation, as the septa with double capillary network are restructured to the mature form of single capillary layer, enhancing the capacity for gas exchange (Burri, 2006)(Figure 2). After this, the lungs undergo marked phase of growth via vascular growth as reflected by the 20-fold increase in alveolar and capillary surface areas from birth to adulthood (Burri, 2006). During childhood, alveolar formation remains at a slow pace, and in adult life, alveolar formation, by septation, may occur in functional recovery situations (Burri, 2006).

Lung morphogenesis is a highly orchestrated process regulated by both genetic and environmental factors (Cornett et al., 2013)tring. Airway and vascular development are closely interactive processes and lung development is dependent on interactions among the epithelium, mesenchyme and endothelium (Shannon and Hyatt, 2004). Cellular processes, signaling mechanisms and transcription factors regulating lung development remain poorly understood (Burri, 2006; Maeda et al., 2007).

The gene expression pattern, analyzed by a microarray method, that controls mouse lung maturation at different time points from the canalicular stage to the saccular stage at birth, is known. Major biological pathways induced during the canalicular stage are cell adhesion, vasculature development, and lipid metabolism, as innate defense and

immune responses are induced at later gestational ages. This suggests that host defense in the lung are critical for survival after birth (Xu et al., 2012). Further, in fetal rat lungs, genes involved in cell proliferation, cell differentiation and development show peaked expressions at days 18 to 20 during the canalicular to saccular stage and from then on their expression decreases and is low in adult lungs. These genes regulate growth factors, plasma membrane receptors, adhesion molecules, intracellular signalling molecules and transcription factors (Weng et al., 2006). Furthermore, in postnatal rat lung fibroblast culture, genes coding transcription factors are upregulated during septation and downregulated at late alveolarization, as the genes coding extracellular matrix components are downregulated at septation and upregulated during late alveolarization and both return to a very low level in adulthood (Boucherat et al., 2007).

2.3.2 Respiratory distress in infants of diabetic mothers

Infants of diabetic mothers are at increased risk for respiratory distress and disturbed cardiopulmonary adaptation (Seppänen et al., 1997; Vela-Huerta et al., 2007). The most complicated maternal diabetes is most frequently associated with respiratory distress (Langer, 2002). The pulmonary problems are explained largely by surfactant deficiency and delayed transepithelial absorption of lung fluid (Kjos et al., 1990; Nold and Georgieff, 2004; Robert et al., 1976; Weindling, 2009). Hyperinsulinemia may delay fetal lung development by inhibiting the normal maturational effect of cortisol on the lung surfactant protein gene expression (Nold and Georgieff, 2004).

Laboratory data indicate that high glucose concentration inhibit surfactant synthesis and secretion by fetal rat type II pneumocytes *in vitro* (Gewolb and O'Brien, 1997) and decrease surfactant protein B and C mRNAs in fetal rat lung explants (Rayani et al., 1999). During the last four days of STZ-diabetic pregnancy, surfactant protein A, B and C mRNAs and the protein contents of A and B are decreased in fetal rat (Guttentag et al., 1992a; Guttentag et al., 1992b). A recent study with mice show that increased insulin signalling through the overactivation of Akt-mTOR signalling in lung epithelial cells *in utero* results in signs of RDS and perinatal lethality in preterm pups (Ikeda et al., 2011).

2.3.3 Genetic changes in fetal lungs in experimental maternal hyperglycemia

Gene expression findings, in the perinatal lungs exposed to maternal hyperglycemia, are limited. In addition to surfactant protein expression changes mentioned above, only a few single genes have altered expression in fetal rat lungs exposed to maternal STZ-induced hyperglycemia: Hox gene *beta* 5, a developmental gene in the lungs, which normally has decreasing expression with advancing gestation, has delayed decline in expression (Jacobs et al., 1998), and nuclear receptor PPARα, a regulator of nitric oxide overproduction in different tissues, has decreased expression simultaneously with increased nitric oxide production (Kurtz et al., 2012).

2.3.4 Morphological changes in perinatal lungs in experimental maternal hyperglycemia

Maternal hyperglycemia delays fetal rat and rabbit lung morphological development during canalicular to saccular stage (at days from 18 to 21 of gestation). The distal airspace walls are thicker, the airspace area is smaller, and mesenchymal cells and glycogen accumulation in the cytoplasm are more abundant. Further, the airspace is lined with more immature coboidal epithelial cells as the amount of both type I and II pneomocytes is decreased and the differentiation of the type II pneumocytes is delayed. The migration of pulmonary capillaries and the fusion of endothelial basement membrane with that of alveolar epithelium are altered (Pinter et al., 1991; Sosenko et al., 1980; Treviño-Alanís et al., 2009). Simultaneously, the Na^+,K^+-ATPase mRNA and activity are reduced in epithelial cells lining the forming alveoli, indicating a decreased ability of active fluid absorption (Pinter et al., 1991). At birth, newborn rat lungs exposed to maternal hyperglycemia show either reduced lung air:mesenchyme ratio and the average size of alveoli (Thulesen et al., 2000) or normal morphology (Pinter et al., 1991). Furthermore, the influence of maternal hyperglycemia on later alveolarization and microvascular maturation of the offspring is demonstrated in rat lungs, as the development of type II epithelial basement membrane is delayed and the density of pulmonary capillaries is reduced from the fetal saccular stage through the eight postnatal day (Grant et al., 1984). Similarly, *postmortem* studies of infants of diabetic mothers show accelerated muscularization in small pulmonary arteries, early in the third trimester, suggesting a disturbance in vascular development (Colpaert et al., 1995) (Table 1).

2.3.5 Postnatal hyperoxia-induced lung injury

Premature neonatal lungs are frequently exposed to high concentrations of supplemental oxygen and this situation may be further aggravated by diminished antioxidative capacity. The detrimental effect of supplemental oxygen in the pathophysiology of bronchopulmonary dysplasia is extensively reviewed, both clinically and experimentally (Bourbon et al., 2009; Saugstad, 2010). Apoptosis and proliferation is altered in distal airways in premature infants exposed to high concentrations of oxygen as well as in various animal models with different oxygen concentrations (Chen et al., 2007; Dieperink et al., 2006; Londhe et al., 2011; May et al., 2004; McGrath-Morrow and Stahl, 2001; Warner et al., 1998; Yee et al., 2006). Hyperoxic exposure have led to alveolar simplification with large, thin-walled terminal airspaces and decreased septation in murine models (Chen et al., 2007; Kunig et al., 2005; Londhe et al., 2011; Warner et al., 1998) and decreased pulmonary vessel density (Kunig et al., 2005). Prolonged hyperoxia, up to day 10, affects pulmonary signaling pathways associated with inflammation, coagulation, fibrinolysis, extracellular matrix turnover, antioxidants, DNA repair, signal transduction and cell cycle regulation in premature rats (Wagenaar et al., 2004) (Table 1).

Table 1. Timing of morphological alterations in lung development during the exposure of maternal hyperglycemia or postnatal hyperoxia in various species

Stage	**Human lung** (Burri, 2006)	**Rat lung** (Pringle, 1986)	**Normal events** (Maeda et al., 2007)	
Prenatal period				
Embryonic stage	3rd-7th weeks	10th-13th days	Formation of the major bronchi	
Pseudoglandular stage	5th-17th weeks	13th-18th days	Branching of bronchi and pulmonary vasculature	
Canalicular stage	16th-26th weeks	18th-20th days	Organization of the pulmonary acinus and pulmonary vascular bed, the onset of pulmonary epithelial cell differentiation and surfactant synthesis	
Saccular stage	24th-38th weeks	20th fetal days to 4th days postnatally	Widening of the peripheral airspaces, differentiation of the respiratory epithelium and surfactant synthesis, increasing vascularity of the saccules	
Postnatal period				
Alveolar stage	36th weeks prenatally to 1-2 years, postnatally	4th-14th days, postnatally	Growth of the alveolar surface area by septation of the alveoli, secondary septa formation, pulmonary microvascular maturation	

	Hyperglycemia-induced alterations in the lungs	**Hyperoxia-induced alterations in the lungs**
	Rats, fetal days 18, 19 and 21 or rabbits, late canalicular / early saccular stage; immature tubular and airspace structures; small thick-walled air spaces, delayed type II pneumocyte differentiation, more abundant glycogen accumulation, pulmonary capillaries more dilated and further from epithelial basement membrane (Pinter et al., 1991; Sosenko et al., 1980; Treviño-Alanís et al., 2009)	Mice, 95% O_2 exposure for fetal days 16-18 and increased apoptosis in distal airways in lung explants (Dieperink at al. 2006)
	Rats, fetal day 21: thick distal airspace walls with more immature coboidal epithelial cells, smaller air spaces, mesenchymal cells more abundant (Pinter et al. 1991) Rats, postnatal day 1: reduced lung air:mesenchyme ratio and the average size of air spaces (Thulesen et al. 2000) or normal morphology (Pinter et al., 1991)	Human, born in weeks 22-36 and ventilated and oxygen-treated: increased alveolar epithelial cell apoptosis and epithelial, endothelial and smooth muscle cell proliferation in post-mortem studies (May et al., 2004) Mice, term, 92% O_2 exposure for days 0-5 and increased apoptosis in peripheral lung (McGrath-Morrow and Stahl, 2001)
	Rats, from the saccular stage to 8 days postnatally: delayed development of type II epithelial basement membrane and reduced density of pulmonary capillaries (Grant et al., 1984).	Rats or mice, term, 75-85% O_2 exposure for days 2-14, days 1-15 or days 1-28: fewer and larger alveolar spaces, decreased alveolar septation, decreased alveolar cell proliferation during the first week, increased collagen deposition after two weeks, decreased vessel density (Kunig et al., 2005)(Londhe et al. 2011)(Warner et al., 1998) Rats or mice, term, 95-100% O_2 exposure for days 1-4 (Yee et al. 2006) or for days 1-7 (Chen et al., 2007); large, thin-walled airspaces with decreased septation; increased apoptosis in airspace walls; diminished type II cell proliferation at day 4 and elevated type II cell proliferation at day 14 after recovery
	Rats, term, 95% O_2 exposure for days 0-5 after maternal hyperglycemic exposure intrauterinally - smaller mean airspace size after the dual exposure than after the hyperoxia alone; decreased number of septal crests after both dual exposure; and hyperoxia alone (Sosenko and Frank 1986)	

3 AIMS OF THE PRESENT STUDY

The objective of this thesis was to investigate the effect of maternal hyperglycemia on developing tissues in both human and experimental settings.

The specific aims were:

I. To investigate the effect of maternal type 1 diabetes on the gene expression profile of the human umbilical cord tissue representing an extension of fetal vasculature.

II. To assess the influence of maternal hyperglycemia on gene expression profile and functional pathways in neonatal rat lungs during late alveolarization.

III. To determine the effects of maternal hyperglycemia on neonatal rat lung structural development and the possible concomitant changes in pulmonary cell apoptosis and proliferation.

IV. To study the combined effect of hyperglycemia priming and postnatal oxygen exposure on neonatal rat lungs.

4 MATERIALS AND METHODS

4.1 Participants in the umbilical cord study (I)

The study (I) included all women who had type 1 diabetes classes from White B to White R (White, 1949) and who underwent elective cesarean section in local/spinal anesthesia between October 2010 and August 2012 in the labor and delivery unit of Turku University Hospital, Finland. The control group consisted of healthy women whom had elective cesarean section with local/spinal anesthesia due to fear of vaginal birth, history of previous cesarean sections or known delivery tract obstruction. Pregnancies complicated by any of the following conditions were excluded from the study: multiple gestation, gestational age less than 36 weeks; smoking during pregnancy; fetal breech position; maternal primary hypertension or preeclampsia; chorioamnionitis; neonatal early onset sepsis and dysmorphic symptoms of the newborn. Data concerning pregnant women were obtained from the records of Turku University Hospital and participating women gave a written consent before cesarean section. The local ethics committee approved the study.

Gestational age was confirmed by ultrasound examination between 11 to 13 weeks and 6 days and all women were further examined ultrasonographically at 18-22 gestational weeks. Gestational diabetes mellitus was excluded at 24-28 weeks of gestation by using 75 g oral glucose tolerance test. Diabetic women were followed by frequent visits in the Department of Obstetrics and the Department of Internal Medicine of Turku University Hospital to optimize antenatal care. Blood glucose levels were regularly controlled by the diabetic women´s own follow-up and by determining the blood glycosylated hemoglobin (GHbA1c) at the hospital visits.

4.2 Umbilical cord tissue samples (I)

The umbilical cord samples were obtained immediately after cesarean section. For microarray analysis, a tissues segment of 1 cm was cut sharply at the distance of 5-7 cm from the baby. Within 10 min, it was flash frozen in liquid nitrogen and stored at -80ºC until RNA extraction. For histological analysis, two 3 mm length segments proximally and distally to the gene expression segment were obtained, immersion-fixed with 10 percent buffered formalin for 24 hours, embedded in paraffin and cut in 3.5 μm thick sections.

4.3 Animals (II-IV)

Animal care was in accordance with the guidelines set by the European Community Council Directions 86/609/EC. The rats were purchased from The Central Animal Laboratory of the University of Turku and housed in pathogen-free conditions. The

Turku University ethics committee for animal experiments and the Animal Experiment Board of Finland approved this study.

4.3.1 Induction of maternal hyperglycemia

Hyperglycemia was induced in female Spraque-Dawley rats before pregnancy with an intraperitoneal injection of 35 mg/kg streptozotocin (STZ; Sigma, St Louis, MO, USA) dissolved in citrate buffer (0.01 mol/l, pH 4.5). Blood glucose levels were measured two days after streptozotocin injection using a Glucometer Elite (Bayer, USA). Rats with a blood glucose level exceeding 15 mmol/l were included in the study. When needed, two additional doses of 15 mg/kg STZ were given at two day intervals until the blood glucose level exceeded 15 mmol/l. Healthy female rats served as controls (III-IV). In the microarray study, a rat with no glycemic response to streptozotocin was used as a normoglycemic control (II). All female rats were caged overnight with a male. The day a positive vaginal smear was obtained was designated as day 1 of gestation. Blood glucose concentration of pregnant hyperglycemic dams was measured at day 1, 8 and 15 of gestation, as well as, at the day of delivery (after delivery). A blood sample from control dams was taken only once on day 1 of gestation. All pups born to hyperglycemic dams (DM pups) (12 litters) and control pups (12 litters) were delivered spontaneously at term (22 days after conception) (Table 2). Their own dams nursed the litters remaining in room air.

Table 2. The total number of rat pups / the number of litters in different study groups: maternal hyperglycemia-exposed pups (DM pups) followed in room air or hyperoxia (85% O_2) and pups of healthy dams (controls) in room air or hyperoxia.

Study group		Controls in room air			DM pups in room air			Controls in 85% O_2	DM pups in 85% O_2
Days, d; Age		d0	d7	d14	d0	d7	d14	d7	d7
Study	Total, pups and litters								
II	8 /2			4 /1			4 /1		
III	211 /18	25 /2	33 /3	55 /4	34 /3	30 /3	34 /3		
IV	175 /16	25 /2	33 /3		34 /3	30 /3		24 /2	29 /3

The same animals were used in both studies III and IV at d0 and at d7 in room air as they were used as controls for hyperoxia-exposed animals in the study IV.

4.3.2 Hyperoxic exposure (IV)

The litters were randomly exposed at day (d) 0 to room air (3 litters of control pups in room air and 3 litters of pups pre-exposed to fetal hyperglycemia, DM pups in room air) or hyperoxia (fraction of inspired oxygen 0.85) (2 litters of control pups in 85% O_2 and 3 litters of fetal hyperglycemia-exposed pups, DM pups in 85% O_2 pups) for 7 days (Table 2). Oxygen exposure was carried out in standard cages placed within a modified

infant incubator. The incubator chamber was left leaky intentionally by not sealing the openings on the wall and roof. A mixture of gas was delivered at rate 5 l/min to maintain the oxygen concentration. This continuous flow prevented the accumulation of carbon dioxide. Temperature, oxygen level and carbon dioxide were controlled twice a day. Once a day, the oxygen chamber was briefly opened and the cages were removed from the chamber to room air for 10 minutes for cleaning. At the same time, the dams were rotated. After replacement, the former equilibrium was achieved within 10 minutes. Room air controls were caged next to the chamber in ambient conditions. Among the room air litters, hyperglycemic dams and control dams nursed their own pups. Litters exposed to hyperoxia were nursed in 24 h cycles by both their own dams and healthy, non-injected, newly delivered surrogate dams, having their litters in room air.

4.4 Lung tissue samples (II-IV)

All pups from the same litter were sacrificed at the same day: at d0, d7, or d14. They were weighed and anesthetized with a rapid intraperitoneal injection of sodium pentobarbital (200 mg/kg). They were randomly selected and the lungs were either processed for histological studies (III, IV), micro-CT studies (III) or weighed and frozen rapidly in liquid nitrogen following storing at -80ºC until used (II-IV). For histological studies, the trachea was cannulated, the chest was opened, and the lungs were *in situ* inflated with 10 percent buffered formalin at a pressure of 15 cm fluid column for 5 min, until fully distended. The trachea was then ligated and the lungs were removed under continuous distending pressure. After 24 h immersion into fresh fixative, the lungs were embedded in paraffin and cut into 4 µm thick sections. For electron microscopy, a small piece (1 mm^3) of intact lung tissue was sectioned from the lateral edge of the left main lobe and fixed with 3 percent phosphate-buffered glutaraldehyde, post-fixed in 1 percent osmium tetroxide, dehydrated and embedded in Epon 812.

4.5 Gene expression analyses of the umbilical cord (I) and the lungs (II)

4.5.1 RNA isolation

The gene expression analyses were performed on 6 human umbilical cords from type 1 diabetic pregnancies and 6 control cords, as well as 4 rat lungs exposed to maternal STZ-induced hyperglycemia and 4 control lungs. Total RNA was isolated from umbilical cord or left lung tissue homogenate by using TissueLyser (Qiagen, Hilden, Germany) (I) or Trizol reagent (Invitrogen, Carlsbad, CA, USA) (II) followed by purification using RNeasy mini kit and RNase-free DNase I (Qiagen, Hilden, Germany). The concentration and the quality of the isolated RNA were measured by spectrophotometry (NanoDrop ND-2000 (I) or 1000 (II) Spectrophotometer, Thermo Fisher Scientific, Wilmington, USA) and automated electrophoresis system (Bioanalyzer, Agilent Technologies, CA, USA (I) or Experion, Bio-Rad Laboratories, CA, USA (II)). Total RNA isolation, processing

and hybridization described below as well as Quantitative Real-time Polymerase Chain Reaction (RT-PCR) analysis (below) were performed in The Finnish Microarray and Sequencing Centre at the Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland.

4.5.2 Microarray hybridization

A total of 200 ng (I) or 300 ng (II) RNA was amplified and biotinylated with Illumina TotalPrep RNA Amplification kit (Applied Biosystems, CA, USA). The concentration and the quality of amplified, biotinylated cRNA were verified by spectrophotometry and electrophoresis.

After purification, 750 ng of cRNA was hybridized to the Illumina Sentrix Human HT-12 v.4 Expression BeadChip microarray (I) or Illumina Sentrix RatRef-12 BeadChip microarray (II) (Illumina Inc., San Diego, USA) representing ~ 47 000 genes (I) or 22 000 genes (II), following a standard protocol of Illumina Whole Genome Gene Expression Direct Hybridization Assay Guide, revision A. Hybridization was detected by using Cy3-Streptavidin (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The array was scanned with BeadArray Reader from Illumina. Expression values were generated with GenomeStudio v.2011.1 software and Gene Expression module v.1.9.0 (I) or Bead Studio 2.3.1 software (II) (Illumina). The primary data are available at the Gene Expression Omnibus Database (http://www.ncbi.nlm.nih.gov/geo/) of the National Center of Biotechnology Information (Bethesda, MD, USA), GEO accession number (GSE51546) (I) or (GSE14307) (II).

4.5.3 Microarray data analysis

Microarray data analysis was performed using R statistical analysis software (Team, 2008) and the related Bioconductor module (Gentleman et al., 2004). The data was normalized using the quantile normalization method and various measures were inspected during the quality control step. Sample correlations between normalized samples were studied using Pearson´s metrics. Hierarchical clustering was produced using Pearson´s metrics and average linkage to visualize the grouping of the samples according to their general expression measurement similarity. Additionally, the sample relation was studied with a Principal Component Analysis (PCA).

The statistical analysis was carried out using the limma package (Smyth, 2005). Differentially expressed genes between the study and the control samples were filtered requiring the satisfaction of the two following criteria; 1) change in the absolute fold change (FC) had to be at least 1.5 (for study I) or at least 1.2 (for study II) and 2) *P*-value had to be smaller than 0.01 (for study I) or smaller than 0.001 (for study II). The thresholds were chosen based on the clustering analysis of the filtered genes: such thresholds were chosen, that the samples were grouped according to their respective sample group and thus giving evidence of the condition-dependent behavior of the selected genes.

Functional enrichment analysis for differentially expressed genes against the Gene Ontology term database was performed using topGO and GOstats packages in R/Bioconductor. Further, the differentially expressed genes were classified into functional GO categories using the Database for Annotation, Visualization, and integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov). Heatmaps for the significantly affected functional pathways were generated with the freely available software GENE-E (http://www.broadinstitute.org/cancer/software/GENE-E/index.html).

4.5.4 RT-PCR analysis

RNA was isolated from the same samples used for the microarray experiment and was quantified and verified as described above. Reverse transcription and amplification into cDNA were done by using M-MLV Reverse Transcriptase RNase H Minus (Promega, Madison, WI, USA). Real-time reactions were performed by an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA) by using 5 ng cDNA as a template, the TaqMan Universal PCR Master Mix, fluorogenic probes (β-actin probe: Oligomer, Espoo, Finland, others: Roche, Indianapolis, IN, USA) and oligonucleotide primers (Oligomer, Espoo, Finland). Probes and primers for the selected genes (I/Table 1, II/Table 1) were designed by using Universal ProbeLibrary Assay Design Center (Roche, Indianapolis, IN, USA). All measurements were done in duplicate in each sample and the mean value of the duplicates was used for quantitation. Housekeeping gene β-actin from the same sample was used as a normalizing control. Data were analyzed with the SDS 2.3 software (Applied Biosystems, CA, USA). Relative expression was calculated from average comparative threshold cycle (CT) values by using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

4.6 Histological analysis of the umbilical cord (I)

Immunohistochemical stainings for myeloperoxidase and leucocyte common antigen (CD45, LCA) were used to identify the activated neutrophil granulocytes (myeloperoxidase) and lymphocytes (CD45) in umbilical cords used for the microarray experiment as signs of inflammation. The deparaffinized sections were subjected to epitope retrieval with BenchMark XT CC1 solutions: standard solution for myeloperoxidase and mild solution for CD45 (VentanaMedical Systems/Roche Diagnostics, Tucson, AZ, USA), and incubated for 32 minutes with mouse monoclonal antibody for myeloperoxidase, clone 59A5, at a titer of 1:100 (Novocastra, Leica Biosystems, Newcastle upon Tyne, UK) or for 20 minutes with mouse monoclonal prediluted antibody for CD45, clone (2B11 & PD7/26) (VentanaMedical Systems/Roche Diagnostics, Tucson, AZ, USA). The stainings were performed with BenchMark XT (VentanaMedical Systems/Roche Diagnostics, Tucson, AZ, USA) using ultraView Universal DAB Detection Kit (VentanaMedical Systems/Roche Diagnostics, Tucson, AZ, USA).

Stained slides were examined blinded to the study groups. For myeloperoxidase and CD45, the amount of positive cells per a grid was counted around the umbilical vein as they existed only in the venous wall, by using a light microscope with a grid of 0.25 mm^2 at x 100 magnification. The results from the grid areas acquired from the same sample were averaged and a significance ($P < 0.05$) between the diabetic and control group was determined by Student´s t-test.

4.7 Histological and biochemical analyses of the lungs (III, IV)

4.7.1 Morphometric analysis

The hematoxylin-eosin (HE) stained, randomly sampled lung sections (one section per pup; n = 8-11 pups per group, no more than four pups per litter) were observed under microscope at x 200 magnification. Ten noncontiguous fields per lung were randomly photographed in a meandering order at a final magnification of 3.81 pixels per micrometer using a Zeiss Axiovert 200 wide-field microscope and AxioCam MRc5 digital camera (Carl Zeiss, Inc.). Regions containing bronchiolar airways or major vessels were excluded from the analysis, but otherwise all regions had equal probability of being selected for analysis. The sampling was generally followed by the main principles described by the Joint Task Force on Quantitative Assessment of Lung Structure of the American Thoracic Society/European Respiratory Society (Hsia et al., 2010).

Mean chord length, as a measure of alveolar size, was quantified using a method previously described (Lum et al., 1990). The digital images were converted to 8-bit grayscale, binarized, inverted, and analyzed using Scion Image software (Scion Corp., Frederick, MD) with a chord length macro (available from the US National Institutes of Health at http://rsb.info.nih.gov/nih-image). Briefly, a grid of parallel lines spaced at 20 μm was added to the images, and the intersections of lung tissue and grid lines were automatically identified. The length of lines overlying alveolar space was then averaged as the mean chord length. To reduce optical and histological processing noise, chords less than 8 μm and greater than 250 μm were excluded. The results from ten images acquired from the same animal were averaged. Total tissue area was quantified from the same binarized images.

The same images were used to assess the mean thickness of saccular/alveolar septa. Straight lines, ~50-70 per field, were drawn perpendicular to the narrowest segments of septa of distal airspaces. The mean length of lines crossing the septa was determined using ImageJ software (National Institutes of Health, Bethesda, MD). Secondary crests were counted from the same images by identifying their characteristic structure as small ridges extending from both sides of the alveolar wall. The number of small arterioles was counted from the same images. They were identified by appropriate morphology as small circular cross-sections (diameter < 30 μm) containing red blood cells in the lumen and without any or thin muscular wall.

4.7.2 Micro-CT analysis of the pulmonary vessels (III)

Three-dimensional imaging with micro-CT was used to quantitate the structure of the pulmonary vasculature in the intact lung (Ritman, 2005)(Shingrani, 2009). For imaging, 14-day old pups in room air were used (7 DM pups and 7 control pups, 2-3 pups per litter). Blood was flushed from the lungs by infusion of heparinized saline through a pulmonary artery catheter placed through an incision in the right ventricle. A heated and brought to 37°C radio-opaque barium-gelatin mixture was infused at constant pressure into the pulmonary artery until the barium-filled vessels were seen over the surface of the lungs and the contrast media was running freely out from the left atrium excision (Langheinrich et al., 2004). Since the barium itself did not enter into the capillary bed, only arterial vessels were studied. The pulmonary artery was then ligated under pressure, and the lungs were subsequently fixed with tracheal instillation of buffered formalin at constant pressure (15 cm fluid column). After fixation, the left main lobe was excised, placed in a formalin-filled tube and immediately micro-CT imaged.

Micro-CT imaging was performed by SkyScan 1072 (SkyScan Kontich, Belgium) using 60 kV X-ray source acceleration voltage, 100 μA current, 6 μm spatial resolution and 0.45 degree rotation angle. Based on the CT-data obtained, spatial distributions and volume ratios of blood vessels were analyzed using CT-Analyzer software (SkyScan Kontich, Belgium). Total lung tissue volume was extracted from overlying formalin using manually defined volumes of interest (VOIs). Global thresholding was used to detect the blood vessels. Further processing of the data allowed the calculation of histograms of blood vessel diameters. To quantify and compare the data from the two study groups, Gaussian curves were fitted to the peaks in the histograms.

4.7.3 In situ detection of apoptotic cells

Terminal transferase-mediated dUTP nick end-labelling (TUNEL), for the detection of apoptotic cells, was performed in paraffin sections. After labeling with antidigoxigenin antibody, the positive staining was visualized with BCIP/NBT (5-bromo-4-chloro-3´-indolyphosphate/ nitro-blue tetrazolium). A distinct color reaction within the cells was regarded to represent DNA fragmentation. TUNEL-positive cells per field were counted from 30 randomly selected, peripheral lung regions for each randomly selected pup, 8-12 pups per group, by using Olympus BX40 microscope (Olympus, Tokyo, Japan) at x 400 magnification.

4.7.4 Electron Microscopy

For ultrastructural analysis, small pieces of Epon-embedded peripheral pulmonary tissue were cut in 1 μm thick sections and stained with toluidine blue. Areas of interest were selected by light microscope and cut in ultrathin sections. Sections were picked up on Formvar-coated copper grids, contrasted by double-staining of lead citrate and uranyl acetate and examined by Jeol JEM 1200 EX electron microscope (Tokyo, Japan).

4.7.5 Immunohistochemistry

For immunohistochemistry, lung paraffin sections were deparaffinized with xylene and rehydrated through graded ethanol and water. Sections were subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer pH 6.0 (for vimentin staining pH 8.4). The endogenous peroxidase was blocked with 3 percent hydrogen peroxide for 10 min and non-specific Ig-binding sites were blocked with normal horse serum (Vector Laboratories, Burlingame, CA) for 1 h at 20°C. Sections were then incubated overnight at 4°C with mouse anti-rat PCNA at a titer of 1:50, mouse anti-human αSMA at a titer of 1:25 (Vector Laboratories, Burlingame, CA) or 16 minutes with mouse anti-vimentin (V9) (Ventana Medical Systems, Tucson, AZ). For PCNA and αSMA, biotinylated horse anti-mouse rat-absorbed secondary antibody and avidin-biotin peroxidase with DAB-nickel chloride (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) were used according to manufacturer's instructions (Vector Laboratories, Burlingame, CA). Tissue slides were counterstained with Mayer´s hematoxylin, dehydrated, cleared and mounted in non-aqueous medium.

For the quantitation of lung tissue cell proliferation, PCNA stained sections were digitally photographed at x 400 magnification by using Axiovert 200M wide field microscope and Zeiss AxioCam MRc5 camera (Carl Zeiss, Göttingen, Germany). The total amount of PCNA-positive cells per wide field was counted from peripheral lung tissue regions. Ten fields for each randomly selected pup, 6-8 pups per group and 2-3 pups per litter, were analyzed. The images were binarized and a total area of lung tissue was measured in each field. Finally, the amount of PCNA-positive cells per unit tissue area was calculated.

Myofibroblast cells were detected by αSMA staining and mesenchymal cells were detected by vimentin staining (IV). Ten random fields of peripheral lung regions were observed under microscope at x 400 magnification searching for qualitative differences between the groups.

4.7.6 Picrosirius Red and Periodic Acid Schiff stainings

Collagen types I-III were localized for all groups through staining of lung paraffin sections by the Picrosirius Red technique. Briefly, sections were deparaffinized with xylene, rehydrated, stained for 60 min with 0.1 percent Sirius red F3B (Sigma-Aldrich, St. Louis, MO) in saturated aqueous picric acid (pH 2.0), washed in acidified water, dehydrated and cleared in xylene. Ten fields of peripheral lung regions for 7 randomly selected pups per group were analyzed. Regions containing bronchiolar airways or major vessels were excluded. Fields were observed under both transmitted and polarized light on an Olympus BX40 microscope (Olympus, Tokyo, Japan) fitted with camera equipment and digitally photographed at x 200 magnification. The polarized light images were converted to gray-scale showing collagen as white pixels in otherwise black field. A number of white pixels per image was quantitated by using ImageJ software (National

Institutes of Health, Bethesda, MD). Respectively, the transmitted light images were binarized and a total area of lung tissue was quantitated. Finally, the amount of alveolar collagen was calculated as the number of white pixels per unit tissue area.

Periodic Acid Schiff technique was used for all groups for detection of glycogen stores in the alveolar regions distal to airways. Briefly, paraffin sections, deparaffinized with xylene, were rehydrated, oxidized in 0.5 percent periodic acid solution for 5 min, stained in Schiff´s reagent for 15 min, counterstained in Mayer´s hematoxylin for 1 min, washed in water, dehydrated and cleared in xylene. Sections were observed under microscope at x 400 magnification searching for qualitative differences in cytoplasmic glycogen stores of septal and acinar regions between the groups.

4.7.7 Oxidative stress parameters in lung tissue (IV)

Frozen lung tissue specimens were obtained from 7-day-old pups exposed to maternal hyperglycemia and/or postnatal hyperoxia and from control pups. The specimen were homogenized in ice-cold (+ 4 C) 0.1 M Tris buffer (100 mg wet weight/mL) with 1 mM EDTA and 10 μM indomethacin, pH 7.4. For the assays of thiobarbituric acid reactive material, tissue homogenates were heated together with a thiobarbituric acid solution (375 mg/mL) in a boiling water bath for 15 min. The tubes were then cooled and the absorbance measured at 535 nm (Bird and Draper, 1984). 8-Isoprostanes in lung homogenates were determined by STAT-8-Isoprostane EIA Kit (Cayman Chemical, Ann Arbor, MI). Glutathione reduced form (GSH) and glutathione oxidized form (GSSG) were measured by Glutathione Assay Kit by Cayman Chemical (Ann Arbor, MI).

4.7.8 Statistical analysis (III-IV)

Results are expressed as mean ± SD. Diagnostic plots for the data were produced to ensure that the model assumptions were satisfied. Statistical significance ($P < 0.05$) between the animal groups in three different time points (d0, d7 and d14) was determined by two-way analysis of variance (ANOVA) and when significant differences were detected, individual comparisons were made by using Bonferroni *posthoc* tests (III). Statistical significance ($P < 0.05$) between the four animal groups in one time point (d7) was determined by one-way ANOVA, and *posthoc* tests were made by Tukey´s Multiple Comparison (IV). Survival rates of the pups were calculated by using the Kaplan-Meier Method (III, IV).

Student´s t-test was used for determining statistical significance ($P < 0.05$) between the two groups in clinical variables, in histological analyses of the umbilical cord (I) and in micro-CT analysis of the neonatal rat lungs (III). Microarray data (I, II) was analyzed by using R statistical analysis software with limma package, as thoroughly described above.

5 RESULTS

5.1 Umbilical cords from infants of diabetic mothers (I)

5.1.1 Characteristics of the mothers

A total of 9, pregnant, type 1 diabetic women who delivered babies during the study period, three of whom were excluded because of preeclampsia, neonatal early-onset septicemia or neonatal neurologic and dysmorphic symptoms. Six control women were randomly selected during the study period.

An elective cesarean section for diabetic women was performed because of fetal macrosomia (2 women), proliferative retinopathy (2 women), both previous reasons (1 woman), and fear of vaginal birth (1 woman). Three women had type 1 diabetes class D (White, 1949) with the onset of diagnosis under 10 years old and mild pre-proliferative retinopathy and three women had class R diabetes with clear proliferative retinopathy without other diagnosed complications. Their diabetes was in moderate balance with GHbA1c levels: 7.2 mmol/l (mean) during the first trimester and 7.1 mmol/l (mean) during the last trimester (I/Table 1). Five diabetic women experienced hypoglycemia during the first trimester. For controls, the criteria for elective cesarean section were fear of vaginal birth (3 women), known delivery obstruction (2 women) and spastic diplegia (1 woman).

The mean age of the women; the mean number of gravidities and parities; and Body Mass Index before pregnancy was similar in both groups (I/Table 1). In the diabetic group, four women suffered from pregnancy-induced hypertension and had antihypertensive medication, two women used levothyroxine for hypothyroidism, two women used antidepressants, and one woman had hyperlipidemia and used asetylsalicylic acid during pregnancy. At gestational week 31 and 35, two diabetic women received antenatal glucocorticoids. In the control group, one woman used citalopram medication for depression during pregnancy (I/Table 1).

5.1.2 Characteristics of the newborns

The mean gestational age at delivery was lower in the diabetic group compared to controls (38 weeks *vs.* 39 weeks and one day) and one infant was born prematurely (36 weeks and 6 days) in the diabetic group (I/Table 1). The birth weight was similar between the groups and only one newborn was macrosomic in the study group. The proportion of male or female infants was similar between the groups. Apgar scores were lower at 1 and 5 minutes in newborns of diabetic mothers but umbilical cord arterial pH values were similar between the groups (I/Table 1). All newborns were transferred to the neonatal

intensive care unit and their median blood glucose level within 2 hours after birth was 2.0 ± 1.0 (range 0.9-3.6). In addition, five infants of diabetic mothers, compared to one control infant, suffered from respiratory difficulties during the first day. Three of the infants of diabetic mothers needed ventilation support (ventilator for few hours or nasal continuous positive air pressure for the first day). Their diagnoses were pneumonia, wet lungs, or surfactant deficiency. Except for a small ventricular septal defect in one infant of diabetic mother, all routine ultrasound findings were normal in the diabetic group (heart, brain, kidneys) and no hypertrophic cardiomyopathy was detected.

5.1.3 Gene expression in umbilical cord tissue

The microarray analysis was performed on 6 study and 6 control umbilical cords. In the microarray data, good reproducibility of the data was shown with sample correlation values between the biological replicates being very high: greater than 0.965 for the control group and greater than 0.963 for the study group. By PCA clustering analysis, the whole, normalized data set revealed a clear trend, which separated the samples according to the respective sample groups.

Of the over 47 000 genes analyzed, 286 genes were differentially expressed in the study group when compared to controls (FC > 1.5 and $P < 0.01$). Of these, 140 genes were upregulated and 146 genes were downregulated. Hierarchical clustering analysis with differentially expressed genes highlighted the difference in the transcriptional profiles between the two groups (I/Figure 1). One sample of the study group was an outlier, which is shown in the heatmap figure (I/Figure 1).

Among the upregulated genes were Bone morphogenetic protein 4 (*BMP4*), *Delta*-like 1 (*DLL1*), Endothelin receptor type B (*EDNRB*) and Prostaglandin-endoperoxide synthase 1 alias cyclooxygenase 1 (*PTGS1; COX1*). Among the downregulated genes were Collagen type VIII *alpha* 1 (*COL8A1*), Matrix metalloproteinase 2 (*MMP2*), Myocyte enhancer factor 2C (*MEF2C*), Notch homolog 4 (*NOTCH4*), Natriuretic peptide precursor B (*NPPB*), phosphodiesterase 5A (*PDE5A*) and Endothelin 1 (*EDN1*) (I/Table 2).

Enrichment analysis with topGO and GOstat packages in R/Bioconductor software organized the most significantly changed pathways occurring in developmental processes: cardiovascular system development (20 genes); organ mophogenesis (28 genes); regulation of nervous system development (18 differentially expressed genes) and morphogenesis of an epithelium (16 genes). In addition, the pathways associated with secretion (22 differentially expressed genes), extracellular matrix (21 genes) and integral to plasma membrane (35 genes) were changed significantly.

DAVID functional annotation clustering analysis revealed that the most significantly enriched GO terms were associated with the downregulated functions: blood vessel development; vasculature development; extracellular matrix; proteinaceous extracellular

matrix; regulation of transmission of nerve impulse and peripheral nervous system development. Upregulated genes were not clearly clustered into specific biological functions when analyzed separately from the downregulated genes. The heatmap figure of the blood vessel development related genes highlights the transcriptional differences between the groups (I/Figure 2).

To validate the microarray data, we analyzed the expression of 11 selected genes by quantitative real-time polymerase chain reaction, using the same samples as in the microarray experiment. These genes were selected to represent a spectrum of significant expression level changes in the microarray. Consistent with the microarray, the expression differences were greater than 1.5 fold for *BMP4, DLL1, EDNRB*, Platelet derived growth factor D and *COX1* (increased expression), and for *PDE5A* and *NPPB* genes (1.5 fold decreased expression) (I/Table 2). All genes were expressed in the same direction as in the microarray analysis.

5.1.4 Histological changes in the umbilical cord

All umbilical cords included three vessels, which were clearly seen in the stained sections. The staining of activated neutrophil granulocytes (myeloperoxidase) and lymphocytes (CD45) showed no difference between the umbilical cords from diabetic pregnancies and control cords ((1.4 ± 0.5 *versus* 7.5 ± 8.0, mean ± SD, myeloperoxidase-positive cells per a 0.25 mm^2 grid and 3.8 ± 3.0 *versus* 3.0 ± 3.5 CD45-positive cells per a 0.25 mm^2 grid, respectively). Venous intima of the two control cords showed increased amount of neutrophil granulocytes in a restricted area (15-20 cells per a 0.25 mm^2 grid) but the amount was not enough to state the diagnosis of funicitis.

5.2 Animal model (II-IV)

5.2.1 Pregnancy outcome and neonatal survival

STZ induced severe hyperglycemia in the treated rats, as expected. Hyperglycemia was constant throughout the pregnancy and lactation (blood glucose 24.6 ± 4.0 mmol/l, mean ± SD). Approximately half (47%) of the hyperglycemic female rats became pregnant and 52 percent of those pregnant rats gave birth while the pregnancy rate and the delivery rate were 85 percent and 100 percent in the controls, respectively. There was no difference in the length of pregnancy between the groups (22 days).

The litter size was similar in the hyperglycemic dams and control dams, 12.8 ± 3.8 and 13.0 ± 2.0 pups/litter (mean ± SD), respectively. Stillborn and pup death rate at d0 was (12/154) in the hyperglycemia-exposed pups and (3/156) in control pups (non-significant difference). The overall survival rate of the DM pups in room air was decreased when compared to the control pups, 86 percent (98/112) *versus* 98 percent (127/130), respectively, $P < 0.05$) (Figure 3). Four pups died in the litter of one hyperglycemic dam

at d5, but thereafter all pups survived. The number of DM pups in room air used for different analyses was 34, 30, and 34 at 0d, 7d, and 14d, respectively and of control pups 25, 33, and 55 at the same time points, respectively (Table 1).

The survival rate at d7 was impaired significantly by maternal hyperglycemic exposure but was not affected by postnatal hyperoxia alone (Figure 3). The survival rate for hyperglycemia-primed hyperoxia-exposed pups was decreased when compared to hyperoxia-exposed control pups (69% (29/42) *vs*. 92% (24/26), DM pups in 85% O_2 *vs*. control pups in 85% O_2, respectively, $P < 0.05$).

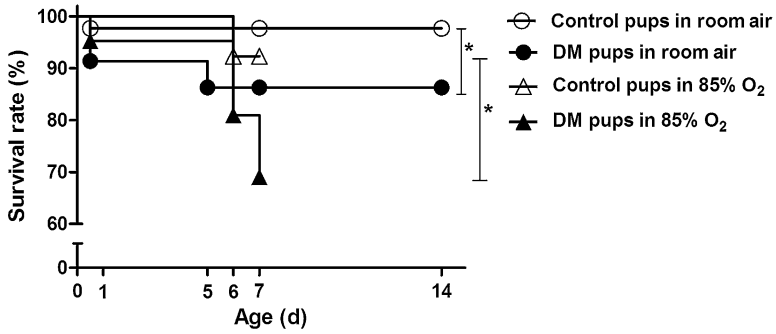

Figure 3. Survival of neonatal rat pups exposed to maternal hyperglycemia (DM pups) followed by room air or hyperoxia (85% O_2) and in pups of healthy dams (control pups) in room air or hyperoxia. The pups found dead during the first day were either stillborn or died within 12 hours after delivery. *$P < 0.05$.

5.2.2 Weight gain

The body weight was not affected by maternal hyperglycemia alone. Hyperoxia-exposed pups had significantly lower body weight than their corresponding air controls at d7 and exposure to fetal hyperglycemia further amplified this effect. Relative lung weight (lung weight to body weight ratio) was decreased significantly in DM pups at d0. Otherwise, it remained unchanged after hyperglycemic and/or hyperoxic exposure (Table 3).

Table 3. The effect of maternal hyperglycemia on neonatal rat weight and lung morphological parameters during two week period postnatally (days 0, 7 and 14) and at one week of age in room air (DM pups in room air) or hyperoxia (DM pups in 85% O_2) when compared to the pups of healthy dams (control pups) in room air or hyperoxia.

	Age, days	Control pups in room air	DM pups in room air		Control pups in 85% O_2		DM pups in 85% O_2	
Body weight (g)	0	5.77 ± 0.25	6.16 ± 0.42					
	7	14.35 ± 0.83	13.05 ± 0.90		12.70 ± 1.7		11.56 ± 1.2	* **
	14	27.33 ± 2.1	26.71 ± 1.7					
Relative lung weight (%)	0	1.75 ± 0.11	1.37 ± 0.03	*				
	7	1.79 ± 0.11	1.80 ± 0.10		1.84 ± 0.28		1.74 ± 0.18	
	14	1.50 ± 0.08	1.37 ± 0.09	*				
Septal thickness (μm)	0	5.35 ± 0.39	4.41 ± 0.38	*				
	7	4.05 ± 0.25	3.76 ± 0.12	*	3.45 ± 0.35		4.32 ± 0.29	** †
	14	3.33 ± 0.13	3.17 ± 0.18					
Alveolar chord length (μm)	0	54.07 ± 5.7	53.38 ± 6.3					
	7	45.92 ± 3.7	42.37 ± 5.4		54.72 ± 4.0	* **	46.60 ± 4.3	†
	14	42.64 ± 4.1	40.42 ± 3.6					
Total tissue area (%)	0	25.94 ± 1.9	23.09 ± 2.8	*				
	7	28.7 ± 2.7	29.32 ± 2.1		23.52 ± 1.6	* **	24.44 ± 1.7	
	14	24.50 ± 1.8	25.11 ± 1.7					
Number of secondary crests per field	0	-	-					
	7	45.35 ± 4.7	48.55 ± 7.2		22.30 ± 3.6	* **	17.60 ± 4.7	* **
	14	49.79 ± 7.5	69.23 ± 8.2	*				
Number of small vessels per field (diameter < 30 μm)	0	10.20 ± 3.0	8.99 ± 2.5					
	7	10.12 ± 1.9	10.51 ± 2.1		8.89 ± 1.5		8.22 ± 1.2	* **
	14	9.90 2.5	10.27 ± 2.4					

At day 0, values are from corresponding control and DM pups sacrificed at d0. Initial body weights of the other pups at d0 were not recorded. Values represent mean ± SD; n = 8-14 rats in each group. *$P < 0.05$ *vs*. control pups in room air at corresponding age; **$P < 0.05$ *vs*. DM pups in room air at d7; †$P < 0.05$ *vs*. control pups in 85% O_2 at d7.

5.3 Gene expression in the lungs exposed to maternal hyperglycemia (II)

The microarray analysis was performed on 4 study and 4 control lungs. In the microarray data, sample correlation values between the normalized expression values of biological replicates were very high: greater than 0.9897 for the control group and greater than 0.983 for the study group, indicating good reproducibility. Of the 22 000 genes analyzed, 227 genes were expressed differentially in the lungs of pups from the hyperglycemic dam when compared to the control pups (FC >1.2 and $P < 0.001$). Of these, 73 genes

were upregulated and 154 genes were downregulated. Hierarchical clustering based on similarity in gene expression using all differentially expressed genes highlighted the difference in the transcriptional profiles between the two groups (II/Figure 1).

The most significantly upregulated gene was collagen triple helix repeat containing 1 (*Cthrc1*) (II/Table 2). Genes for collagen type I *alpha* 1 (*Col1a1*), collagen type III *alpha* 1 (*Col3a1*) and matrix metalloproteinase 14 (*Mmp14*) were also significantly upregulated. Genes for insulin-like growth factor binding protein 2 (*Igfbp2*), platelet derived growth factor receptor alpha (*Pdgfra*), endothelin 1 (*Edn1*), glutathione peroxidase 3 (*Gpx3*) and superoxide dismutase 3 (*Sod3, EC-SOD*) were significantly downregulated.

In the DAVID functional annotation tool, the most significantly enriched biological functions among differentially expressed genes, passing by Fisher exact test *P*-value of less than 0.001, included extracellular region and extracellular matrix (upregulated) and cell proliferation, extracellular region, cell adhesion and oxygen and reactive oxygen species metabolism (downregulated) (II/Table 3).

5.4 Newborn lungs exposed to maternal hyperglycemia (III)

5.4.1 Morphology

Postnatal thinning of alveolar walls was found in all pups in room air. However, alveolar septa were thinner significantly in the DM pups compared to controls at birth and at d7 (Table 3, Figure 4), which was also confirmed by electron microscopy (data not shown). Lung total tissue area was smaller in DM pups at d0 in accordance with the finding of thinner septa. A trend towards an increased number of secondary crests in DM pups was found already at d7 and they were increased significantly at d14. The thickness of alveolar walls did not differ between the groups at the late stage of alveolarization (d14). Alveolar size (chord length) or the number of small-sized pulmonary vessels was not affected by maternal hyperglycemia (Table 3, Figure 4).

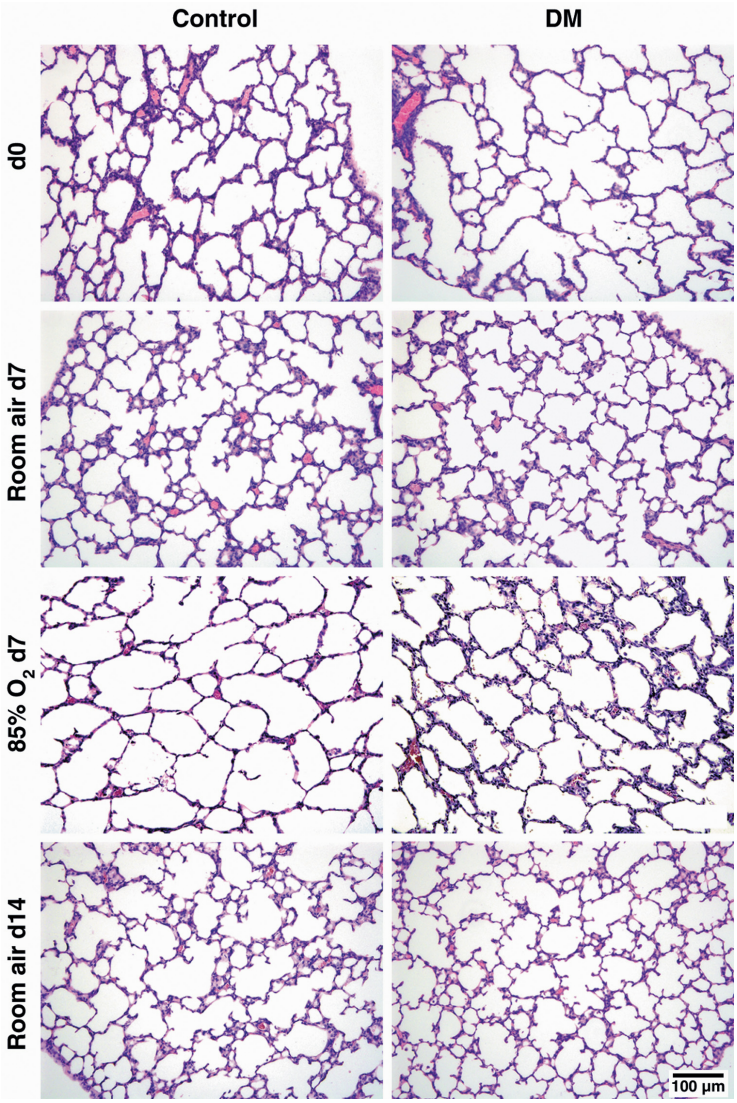

Figure 4. Morphology of representative lung sections from control pups (left) and pups exposed to maternal hyperglycemia (DM, right) at the age of 0, 7 or 14 days in room air and the effect of hyperoxia exposure (85% O_2) on lungs at the age of 7 days. HE staining; magnification 100; scale bar 100 µm.

5.4.2 Micro-CT examination of pulmonary arteries

Micro-CT analysis with contrasting agent was used to investigate the differences in the structure of pulmonary arterial vasculature between the DM and control pups in room air at d14. Arterial volume ratios and spatial distribution measurements were similar between the groups (III/Table 2A). Arterial diameter ranged from 15 µm (smallest visible arterioles limited by resolution) to 750 µm in both groups and the mean diameter of all pulmonary arteries was similar. Neither the number of arteries per unit tissue area nor the mean distance between the arteries differed between the groups. Additionally,

no difference was shown in the fraction of different sized small arteries, diameter 15-66 µm, between the groups (III/Table 2B). Two-dimensional arteriograms and histological sections as well as three-dimensional images revealed homogenous filling of the pulmonary arterial tree after infusion of barium mixture (Figures 5 and 6).

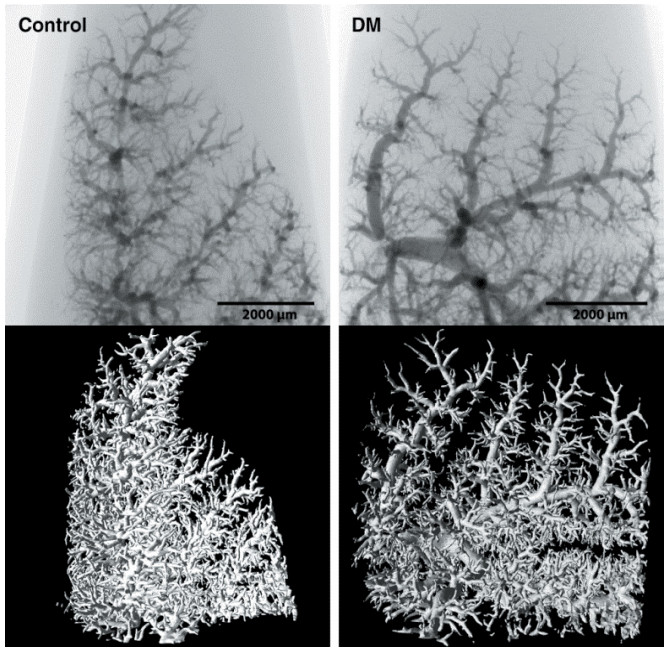

Figure 5. Barium-filled pulmonary arteriogram and the corresponding three-dimensional reconstruction (below) of the left main lobe of 14-day old control and DM pup imaged by micro-CT (figure from study III).

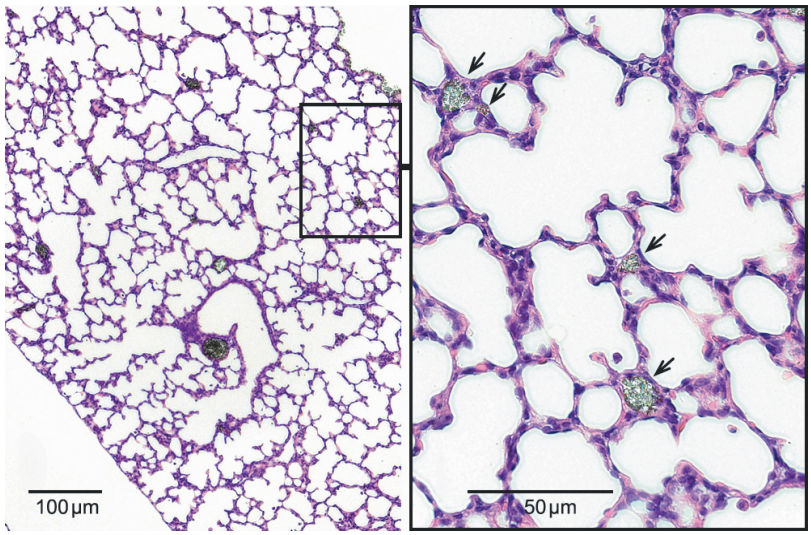

Figure 6. Representative HE-stained section and the insert (right) of the lungs showing proper barium filling of the pulmonary arterioles.

5.4.3 Apoptosis and proliferation

TUNEL-positive cells, indicating the presence of apoptotic cell death, showed characteristic chromatin condensation in the nuclei. Cellular apoptosis was also confirmed by electron microscopy (data not published). Maternal hyperglycemia resulted in enhanced pulmonary cellular apoptotic peak at birth (Figure 7A). At d7 and d14, no significant differences were found between the groups in room air. In DM pups in room air, the number of TUNEL-positive cells decreased during the first week of life and then slightly, although not significantly, increased at d14. In control pups, no significant differences were found at d0, d7 and d14. Although the specific cell types could not be identified, the positive cells were mainly lining alveolar walls indicating epithelial cell involvement.

Maternal hyperglycemia resulted in increased number of PCNA-positive cells, representing increased pulmonary cell proliferation, during the whole study period (at d0, d7 and d14) in room air (Figure 7B). In DM pups, PCNA-positive cells were located more intensively in alveolar septa, at the base and tips of secondary septa and on acinar areas.

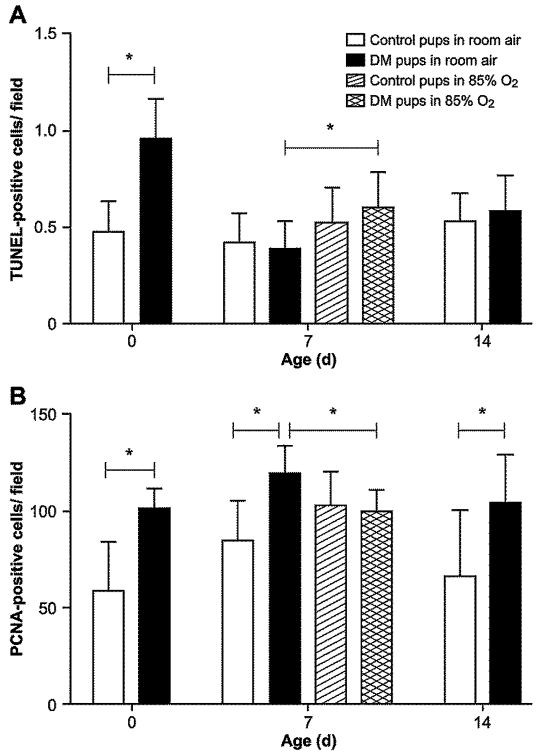

Figure 7. The number of TUNEL positive cells (A) and PCNA-positive cells (B) in neonatal rat pups exposed maternal hyperglycemia (DM pups) followed by room air or hyperoxia (85% O_2) and in pups of healthy dams (control pups) in room air or hyperoxia. Values represent mean ± SD. n = 8-12 (TUNEL) and 6-8 (PCNA) rats in each group. *$P < 0.05$.

5.4.4 Myofibroblasts

The number of αSMA-positive cells, representing myofibroblasts, was not affected by maternal hyperglycemia (data not published). Positive cells were seen in all groups and most abundantly they were seen at d7 corresponding to the stage of secondary septation. They were located mainly at the ridges of arising secondary septa or at their tips, as well as, within the smooth muscle underlying vessels and bronchi.

5.4.5 Collagen and glycogen

Collagen types I-III were stained by Picrosirius Red in paraffin fixed lung tissue sections. When observed in transmitted light, a slight but non-significant increase was found in the accumulation of collagen with advancing age, which did not lead to differences between the groups at any time points. The negative result was confirmed quantitatively by using polarized light under which stained collagens appeared white in gray-scale images (data not published).

Pulmonary glycogen stores were determined by Periodic Acid Schiff staining. Glycogen granules (~0.5-2.5 μm) were observed mainly within the cytoplasm of interstitial cells and, occasionally, within alveolar septa. At d0, glycogen granules were seen equally in the lungs of DM and control pups. At d7 and at d14 in room air, glycogen granules remained abundant in DM lungs and tended to decrease with advancing age in control lungs (data not published).

5.5 The effect of hyperoxia on postnatal lung development (IV)

Hyperoxia alone impaired pulmonary development as indicated by decreased septal wall thickness and increased alveolar chord length at d7 in control pups when compared to control pups in room air (Table 3, Figure 4). Accordingly, lung total tissue area and the number of secondary crests were also decreased at d7 in control pups in 85 percent O_2 when compared to control pups in room air (Table 3, Figure 4). The number of small arterioles between the control pups in 85 percent O_2 and the control pups in room air did not differ (Table 3). Furthermore, hyperoxia alone did not affect cell proliferation or apoptosis in the lungs of control pups at d7 (Figure 7).

5.6 Neonatal lungs exposed both to maternal hyperglycemia and postnatal hyperoxia (IV)

5.6.1 Morphology

Hyperoxia inhibited the physiological septal wall thinning at d7 in DM pups when compared to DM pups in room air (Table 3, Figure 4). In fact, the septal thickness of DM pups in 85 percent O_2 at d7 corresponded to the septal thickness of DM pups at

d0. Furthermore, hyperoxia in DM pups did not affect alveolar chord length or lung total tissue area when compared to room air controls (Table 3, Figure 4). However, the number of secondary crests and the number of small arterioles were decreased in DM pups in 85% O_2 at d7 when compared to room air controls (Table 3). The results are summarized in Table 4.

5.6.2 Apoptosis and proliferation

Hyperoxia resulted in increased pulmonary cell apoptosis and decreased pulmonary cell proliferation at d7 in DM pups when compared to DM pups in room air (Figure 7). However, no differences in proliferation or apoptosis were observed between the two hyperoxia-exposed groups.

5.6.3 Collagen, glycogen, myofibroblasts and vimentin

To explain the finding of thick alveolar walls and inhibited alveolar wall thinning in hyperoxia-exposed, hyperglycemia-primed pups, we stained the lung tissue sections with Picrosirius Red and Periodic Acid Schiff to show collagen and glycogen but did not find any difference in their deposition between the study groups (data not shown). However, staining with vimentin (type III intermediate filaments) demonstrated that the thick airway walls were filled by cells of mesenchymal origin (IV/Figure 5). Since the staining of myofibroblasts by αSMA did not differ between the study groups (data not shown), we presumed that these vimentin-positive mesenchymal cells were lipofibroblasts.

5.6.4 Electron microscopy

To identify the mesenchymal cell type in the thick airway walls, we performed an electron microscope analysis that demonstrated that the airway walls were filled with lipid interstitial cells, lipofibroblasts, in DM pups during hyperoxia. The cells were recognized by lipid droplets within their cytoplasm (IV/Figure 4). Sparse lipid interstitial cells were found in the septa of other groups compared to DM pup lungs and the characteristic clusters of lipid droplets were absent from the septa of control pups exposed to hyperoxia.

5.6.5 Oxidative stress

The biomarkers of oxidative stress, thiobarbituric acid reactive material and 8-isoprostanes, were not affected either by the fetal hyperglycemic exposure or by postnatal hyperoxic challenge at d7. Glutathiones, GSH and GSSG, were slightly, but not-significantly, increased after the combined exposure of hyperglycemia and postnatal hyperoxia, but the ratio of GSH to GSSG was not affected (IV/Figure 6).

Table 4. Summary of the findings in the present dissertation: maternal diabetes-induced changes in human umbilical cords and maternal hyperglycemia and/or postnatal hyperoxia-induced changes in neonatal rat lungs.

Umbilical cord	**Maternal diabetes-induced alterations**
At birth	Downregulated blood vessel development- and extracellular matrix -related genes Altered expression of genes related to the regulation of vascular tone: vasodilatation-favored expression profile
Neonatal rat lung	**Maternal hyperglycemia and/or postnatal hyperoxia-induced alterations**
At day 0, saccular stage	**Maternal hyperglycemia-induced alterations:** Thinner saccular walls Decreased lung total tissue area and relative weight Increased apoptosis and cell proliferation in airspace walls
At day 7, alveolar stage	**Maternal hyperglycemia-induced alterations:** Thinner septa Increased cell proliferation in alveolar walls
	Postnatal hyperoxia alone: Thinner airspace walls Large air spaces Decreased septation
	Dual exposure: Thick alveolar walls – inhibited septal thinning Decreased number of secondary crests Decreased number of small pulmonary vessels Abundant lipid interstitial cells in the alveolar walls Increased apoptosis and diminished cell proliferation in alveolar walls Decreased survival of the pups
At day 14, alveolar stage	**Maternal hyperglycemia-induced alterations:** Upregulated extracellular matrix-related genes Downregulated cellular proliferation- and antioxidant-related genes Increased number of secondary crests Increased cell proliferation

6 DISCUSSION

6.1 Methodological considerations

6.1.1 Microarray analysis

Since the umbilical cord provides an extension of the fetal vasculature, we decided to investigate its biological responses to maternal diabetic milieu in humans. The microarray analysis provided a comprehensive overview of mRNA levels that represented significantly up- or downregulated genes in human umbilical cord exposed to maternal type 1 diabetes. We used the whole umbilical tissue for the analysis because it mainly contains the vessel structures and perivascular stromal cells that are suggested to participate in regulation of umbilical cord blood flow (Can and Karahuseyinoglu, 2007), and thereby affect fetal development. In order to avoid possible acute gene expression changes related to the induction of birth, the umbilical cords were obtained from the pregnancies terminated by elective cesarean section. Similar to our study, the whole umbilical cord tissue was successfully used for genetic examinations in pregnancies complicated with intrauterine growth restriction (Hussain et al., 2008; Lim et al., 2012).

In contrast to umbilical cord tissue, neonatal rat lung tissue, used in our other microarray studies, was a more heterogenic structure containing bronchial trees and vessels between the lung parenchyme and as a result, most of the genes in the array are likely to be expressed by many cell types. Nevertheless, our whole tissue approaches were still useful to evaluate changes in biological signaling pathways and the possible synergic effect of diverse gene expressions on various biological pathways controlling tissue development. In fact, due to the strictly hierarchical structure of signalling and metabolic networks, the biological consequences may be amplified if several mediators in a signal transduction cascade are similarly affected. We further decided to study the lung tissue from two week-old pups to reveal postnatally apparent effects of maternal hyperglycemia on biologic interactions.

Due to post-translational modifications, such as phosphorylation, the observed changes in gene expression level may not necessarily be equivalent to actual protein levels. We did not use the protein measurements or *in situ* hybridizations to detect the actual level and localization of the proteins, which may, to some extent, limit the extrapolation of the gene expression results to the biological functions. Further, we could not specify the cell types having altered gene expression, although many genes of interest are known to be expressed by endothelium, smooth muscle cells, fibroblasts or type II pneumocytes.

6.1.2 Streptozotocin model of maternal hyperglycemia

Animal studies are critical in understanding the pathophysiology of maternal diabetes-induced fetal organ alterations throughout pregnancy and after birth (Jawerbaum and White, 2010). Although the animal models only mimic the clinical disease in humans and the findings are valid for the experimental studies only, they still offer a first-stage approach in order to evaluate how to prevent maternal diabetes-induced alterations. We used an animal model of STZ-induced maternal hyperglycemia, which is widely and successfully used to model human diabetes in pregnant women (Jawerbaum and White, 2010). STZ, a N-nitrose derivative of glucosamine, which was first isolated as a new antibiotic in 1956, causes a dose-related and highly specific damage to pancreatic β-cells, which leads to insulin deficiency (Junod 1969)(Yamamoto 1981)(Schnedl 1994). STZ, itself, is unlikely to be associated with any fetal lung toxicity because its cellular uptake mechanism, glucose transporter 2, is lacking in the lungs (Schnedl 1994)(Thulesen 2000). Further, because of the short half-life of STZ (<6 hours), the direct effect of STZ on fetal organs was avoided by administering it before mating, as recommended by the NIH task force (Farrell 1982).

Our target was to investigate the effects of overt prolonged maternal hyperglycemia on neonatal rat lungs. Thus, we chose to use STZ, which is shown to generate a maternal diabetic state related to severe, uncontrolled type 1 diabetes during pregnancy (Jawerbaum and White, 2010). The level of maternal hyperglycemia and the consequent metabolic fetal effects vary with the dose of STZ. A low dose of STZ is associated with fetal β-cell hyperstimulation, fetal hyperinsulinemia and macrosomia, which mimics gestational diabetes (López-Soldano, Ferrera, 2003)(Pitkin and Van Orden, 1974). Higher doses, on the other hand, induce severe maternal hyperglycemia with sometimes decreased fetal growth rather than macrosomia, which is explained by maternal catabolic state, reduction *in utero* placental blood flow, and fetal pancreatic β-cell collapse, eventually leading to fetal hypoinsulinemia and growth retardation (López-Soldano, Ferrera, 2003)(Pitkin 1974)(Kervran 1978)(Giavini 1986)(Thulesen 2000)(Amri et al., 1999; Caluwaerts et al., 2003).

In this study, we induced a moderate to severe state of maternal hyperglycemia (blood glucose 24.6 ± 4.0 mmol/l) during pregnancy. We did not measure the insulin concentrations of the fetal rats and neither controlled the glycemic state of the newborn pups. However, previous experimental studies comparing different doses of STZ show that a similar stage of maternal hyperglycemia, as seen in our study, may induce different profiles of fetal insulin levels, like hypoinsulinemia (Kervran et al., 1978; López-Soldado and Herrera, 2003), normoinsulinemia (Aerts and van Assche, 1977; Caluwaerts et al., 2003) and hyperinsulinemia (Mulay et al., 1983). On the other hand, after similar degree of maternal hyperglycemia, the neonatal level of blood glucose may decrease during the first day of life from fetal hyperglycemia to neonatal normoglycemia (Aerts and van Assche, 1977). Thus, marked neonatal hypoglycaemia in our model is not likely to be present. Further, maternal hyperglycemia did not affect the pup birth weight in our

model. This is at least partly explained by the level of maternal hyperglycemia with varying metabolic fetal effects. The lack of obvious neonatal macrosomia may also be due to the short pregnancy time in the rat, differences in the percentages of adipose tissue in rat fetuses (1%), human offspring (16%) and the greater weight gain in the human species (Jawerbaum and White, 2010). In fact, this lacking effect of maternal hyperglycemia on the pup body weight is in line with the data from previous similar rat-based experimental studies (Aerts and van Assche, 1977; Jawerbaum and White, 2010; Mulay et al., 1983).

The hyperglycemic dams in our studies were allowed to nurse their own pups, despite of the theoretical possibility that the feeding would compromise their wellbeing. However, regardless of severe hyperglycemia, the dams nursed their pups well, as demonstrated by the similar body weight gains between the DM pups and controls.

Maternal hyperglycemia affected the fertility and delivery rate in our study, which was not surprising when considering the metabolic state of the dams. However, litter size and the length of pregnancy was similar between the diabetic rats and healthy controls. Similar results have been reported in previous studies with corresponding hyperglycemic stage (Mulay, 1983)(Amri, 1999)(Aerts and van Assche, 1977; Kervran et al., 1978; Thulesen et al., 2000). Stillborn pups and the pups which died shortly after delivery (within 12 hours) were found more frequently in the DM group than controls but the difference was not significant. Due to practical and technical reasons, we were not able to study these succumbed pups. Since these pups were mainly delivered at night, proper handling and reliable examination of the lung structure was not possible. Nevertheless, the pulmonary findings of these DM pups were likely to be more aberrant than those of the studied DM pups and therefore we consider our results to be representative for the whole DM group.

In our microarray study of neonatal rat lungs, we used the control dam, which was injected with same dose of STZ as the hyperglycemic dam, but remained normoglycemic without any glycemic response. Following the glucose levels through the pregnancy ensured this. The main reason for this procedure was to focus on the effects of overt maternal hyperglycemia on pulmonary gene expression and, secondly, to standardize the unknown influences of STZ on the gene expression profile. Further, in this microarray study, we used the offspring of only one dam per group, as our primary aim was to generate new directions for the future studies. This litter effect may, however, be a limitation in this study and must be taken in consideration when interpreting our results.

6.2 Gene expression in human umbilical cord in diabetic pregnancy

We hypothesized that the diabetic environment would induce multiple gene expression changes in the umbilical cord, similar to alterations in the placental vascular phenotype (Leach, 2011), and affect primarily the pathway associated with vascular development.

Indeed, previous studies indicate that umbilical cords from diabetic pregnancies may have structural modifications, such as changes in extracellular matrix and vascular modeling (Asmussen, 1980; Cromi et al., 2007; Singh, 1986). Similar vascular alterations may have been developed also in other fetal vascular beds when exposed to hyperglycemia (Leach, 2011). Indeed, the maternal hyperglycemic environment induces embryonal vasculopathy in murine tissues (Grant et al., 1984; Larger et al., 2004; Pinter et al., 2001; Pinter et al., 1999; Sosenko et al., 1980) and leads to vascular dysfunction in adult offspring (Holemans et al., 1999; Wichi et al., 2005). As expected, our microarray data from umbilical cords revealed major diabetes-induced gene expression changes in the vascular development pathway. This pathway was mainly affected by downregulation of 15 genes, including extracellular matrix genes that participate in the degradation of the basement membrane, cytokines and growth factors that regulate vascular stability and signal transduction genes associated with angiogenesis. The downregulated extracellular matrix-associated genes *COL8A1* and *MMP2* are suggested to play a role in maintaining vessel wall integrity and structure (COL8A1) (Plenz et al., 2003) and in the initiation of angiogenesis by breaking down the extracellular matrix barrier (MMP2) (Mauro et al., 2010). Downregulated *MEF2C* is an additional vascular wall stabilizer expressed in the developing endothelial cells and smooth muscle cells (Lin et al., 1998)1998. The downregulation of the *NOTCH4* gene and upregulated *DLL1* gene indicate alterations in endothelial Notch signaling pathway, which may reflect insufficient sprouting of angiogenesis in embryonic development (Kume, 2012). One of the most significantly upregulated genes in our present study was *BMP4*, an extracellular signaling protein of the transforming growth factor *beta* superfamily, which is shown to play a role in maternal hyperglycemia-induced cardiac tissue apoptosis in murine embryos (Wentzel et al., 2008) and may inhibit angiogenesis via apoptosis of the endothelial cells (Moreno-Miralles et al., 2009). The significance of these gene expression changes in maternal diabetes-exposed umbilical cord tissue remains uncertain but may be associated with altered perivascular remodeling. This would result in disturbances both in the early sensible and later stabilizing stages of fetal angiogenesis.

Infants of diabetic mothers are frequently prone to delay in immediate postnatal cardiopulmonary adaptation (Seppänen et al., 1997; Vela-Huerta et al., 2007). Especially the closure of the *ductus arteriosus* may be delayed and the pulmonary artery pressure may remain persistently elevated during the first days of life (Seppänen et al., 1997). Our data indicate that a number of genes involved in the regulation of endothelial function were differentially expressed in the umbilical cords from diabetic pregnancies. The simultaneous downregulation of genes of vasoconstrictor substances (*EDN1* and *PDE5A*) together with upregulation of genes of vasodilatation mediators (*EDNRB* and *COX1*) seem to reflect a shift in the gene expression profile towards vasodilatation, thus promoting adequate blood flow to the fetus. As an exception, gene expression of a well-known vasodilator *NPPB*, also known as proBNP, was decreased in our study. ProBNP is released by ventricular cardiac myocytes, but it is also expressed within the decidua of the mouse placenta, where it acts as a vasodilator in the fetal-placental vasculature

(Cameron and Ellmers, 2003). Taken together, alterations in genetic pathways regulating blood flow and vascular tone in diabetic pregnancies could hence contribute to the delay in immediate cardiopulmonary adaptation together with the clinically observed respiratory difficulties, in line with our studies. The observed alterations in endothelial function-related gene expression profile in these infants may produce phenotypic modifications and long-term vascular consequences (Marco et al., 2012; Simeoni and Barker, 2009), which remains to be studied.

6.3 Gene expression in postnatal rat lungs after exposure to maternal hyperglycemia

The first two weeks of life is a critical phase for alveolarization in murine lungs (Burri, 2006) and genes regulating alveolar development show a changing expression profile during different developmental stages (Boucherat et al., 2007b). During the early active phase of alveolarization, genes associated with extracellular matrix formation show decreased expression in rat lung fibroblasts but are then upregulated at late alveolarization and return to a very low level in adult lungs (Boucherat et al., 2007b). Similarly, the expression of the collagenase *Mmp14* gene is increased during the normal lung maturation at postnatal day eight and decreases thereafter, suggesting a crucial regulatory role for it during alveolarization (Boucherat et al., 2007a). At late alveolarization in rat pup lungs exposed to intrauterine hyperglycemia, we found simultaneous upregulation of collagen *alpha* I and III and collagenase *Mmp14* genes, possibly reflecting a perturbation in extracellular matrix protein balance in these lungs. We did not, however, find increased collection of collagen in our histological analyses, which suggests some degree of translational or post-translational modifications.

The most significantly downregulated pathways in our study were associated with cell proliferation and oxidative stress-related genes. In agreement, studies of embryonal tissues show aberrant expression profiles of genes coding cell proliferation and oxidative stress in association with maternal hyperglycemia (Fu et al., 2006; Jiang et al., 2008; Pavlinkova et al., 2009; Reece et al., 2006; Vijaya et al., 2013). Cell proliferation together with apoptosis and oxidative stress are, in fact, widely proposed to be involved in the pathophysiology of diabetic embryopathy (Moley, 2001; Ornoy, 2007; Reece, 2012; Zabihi and Loeken, 2010). Whether the gene expression changes, in our study, are permanent aberrations that affect pulmonary growth and function or are a reflection of temporary disturbed signaling, remains to be elucidated. It is noteworthy that these gene expression changes may be either primary changes initiated by intrauterine hyperglycemia *per se* or secondary compensatory postnatal changes.

During late pregnancy, hyperglycemia together with hyperinsulinemia may induce delayed fetal lung surfactant synthesis, as described earlier. A few single genes mainly related to surfactant proteins have been shown to be altered in fetal rat lungs exposed to hyperglycemia (Guttentag et al., 1992a; Guttentag et al., 1992b; Jacobs et al., 1998;

Kurtz et al., 2012; Rayani et al., 1999). Our present gene expression data extend these observations into the postnatal lungs of offspring of hyperglycemic rats and showed a deviant expression profile of proliferation and oxidative stress-related genes during this critical phase of neonatal lung development. The metabolic derangements during intrauterine hyperglycemia may thus cause changes in gene expression and epigenetic changes of prenatal tissues, including the lungs, producing long-term functional and structural changes.

Prolonged postnatal hyperoxia affects the signaling pathways of premature rat lungs in part as in our postnatal hyperglycemia-exposed lung model, including genes regulating extracellular matrix turnover, antioxidant balance and cell cycle (Wagenaar et al., 2004). On the other hand, collagen *alpha* I and III genes were upregulated in our hyperglycemia lung model of two-week old rats but were downregulated in postnatal rat lungs exposed to hyperoxia (Wagenaar et al., 2004). In addition, the *EDN1* gene was downregulated in both our microarray studies of umbilical cord tissue and postnatal rat lungs exposed to hyperglycemia, but was increased in postnatal rat lungs in hyperoxia (Wagenaar et al., 2004). It is in fact possible that since the hyperoxic exposure results in severe lung injury with disturbed alveolar development and fibrotic collagen accumulations (Kaarteenaho-Wiik et al., 2004; Wagenaar et al., 2004), its gene expression may, as a result, finally turn into downregulation. In our study, conversely, the two-week-old rat lungs exposed to maternal hyperglycemia may attempt to balance on the phenotype of accelerated cell turnover and secondary crest formation, as discussed below. In addition, upregulation of vasoconstrictor *Edn1* in the hyperoxia model may contribute to the elevated pulmonary vascular resistance and pulmonary hypertension frequently seen in hyperoxia-induced lung injury (Gao and Raj, 2010) but its downregulation in our hyperglycemia lung model is more in line with the normal pulmonary adaptation because the production gradually decreases in low pulmonary vascular resistance normal maintenance in newborns (Gao and Raj, 2010).

6.4 Neonatal rat lungs after exposure to maternal hyperglycemia

While the bulk of alveolarization occurs between the 4th and 14th day of life in rats, the thinning and maturation of the distal airspace walls begin already at the first day of life or even slightly before birth to optimize the gas exchange after birth (De Paepe et al., 1999; Kresch et al., 1998). In our model, maternal hyperglycemia resulted in thinner saccular walls in newborn rat pup lungs at birth compared to controls. This was associated with decreased lung relative weight, decreased lung total tissue area, and increased cellular turnover with apoptotic peak and enhanced proliferation in the lining of saccules. Interestingly, our results are in contrast to earlier studies in which maternal hyperglycemia resulted in a tendency for delayed pulmonary development (Thulesen et al., 2000) at birth or normalized morphology after delayed development during fetal period (Pinter et al., 1991). The results are not, however, directly comparable because the previous studies used different time points (Grant et al., 1984), morphometric

methods (Grant et al., 1984) or did not overall evaluate the gross morphology and later development of the lung (Pinter et al., 1991; Sosenko et al., 1980; Thulesen et al., 2000; Treviño-Alanís et al., 2009). In addition, the morphological maturation of the lung may differ from the biochemical maturation. It is also theoretically possible, that differences in lung compliance between our study groups may have influenced the lung inflation during the fixation process, and concomitantly, lung distension and alveolar chord length (Hsia et al., 2010). However, we did not find any signs of incomplete distension and fully inflated lungs with flat pleura and open alveoli were regularly observed in histological sections. Although we measured lung weight as wet weight instead of dry weight and differences in fluid retention could not be calculated, no signs of excess pulmonary tissue fluid in lung histological examination or clinical signs of respiratory difficulties in the pups were found. Altogether, the thinner saccular walls together with reduced lung tissue area, in our model, imply premature structural remodelling, which may lead to deviated lung growth.

The intrauterine hyperglycemic exposure had a prolonged effect on postnatal pulmonary development, since septa remained thinner up to the age of one week, the number of secondary crests was further increased at two week of age and cell proliferation was stimulated during the whole alveolarization period. In agreement, the hyperglycemic fetal exposure resulted in long-lasting changes in type II epithelial basement membrane development and reduced the density of pulmonary capillaries in the fetal saccular stage lungs through the eighth postnatal day in rats (Grant et al., 1984). Together, these results are in line with our microarray findings of altered gene expression still at two weeks of age and further, they may reflect the metabolic memory phenomena and a persisting effect of fetal hyperglycemia on postnatal growth control mechanisms. In contrast to our hypothesis and to the results of previous study (Grant et al., 1984), we did not find any difference in arterial density between the hyperglycemia-exposed lungs and control lungs in either micro-CT imaging at two weeks of age or histological examination from birth to the age of two weeks. As described in detail earlier (III), the technical and timing parameters may limit the comprehensiveness of the micro-CT analysis, as the small vessel size and the vulnerability of the lung tissue limited the imaging of lung vasculature to two-week-old pups. Taken together, our findings suggest that mechanisms leading to postnatal pulmonary remodelling in DM pups are not associated with vascular aberrations detectible with our methods.

The normal alveolarization process is associated with an increase in apoptosis, which leads to the thinning of alveolar septa in order to optimize the gas exchange after birth (Bourbon et al., 2009). In our study, fetal hyperglycemia enhanced this process and resulted in thinner septa in DM pups than in controls during the first postnatal week. This accelerating effect of embryonal hyperglycemia on apoptosis has previously been reported in chicken embryos and mouse embryonic neural stem cells (Fu et al., 2006; Larger et al., 2004). In the normal structural maturation of the lungs, increased apoptosis is found again during the third postnatal week, as the reduction of the number

of fibroblasts and type II cells by apoptosis occurs (Schittny et al., 1998). Concomitantly with increased apoptosis, we found accelerated cell proliferation in DM pup lungs already at birth, possibly reflecting high cell turnover after the embryonal period (Fu et al., 2006; Larger et al., 2004). During the first two postnatal weeks, cell proliferation was high and an abundance of proliferative cells in DM pup lungs were mainly located at the bases and tips of secondary septa and on acinar areas, which are known to be critical for alveolarization. Similarly, the number of secondary crests was increased in DM pups at two weeks of age. At this time point, however, the pathway controlling cell proliferation was downregulated in DM pup lungs. This may viewed as an attempt to balance between apoptosis and proliferation because the normal structural lung development is associated with decreased proliferation of type II cells at two weeks of age (Yee et al., 2006). Future studies are, however, clearly needed to explore the effects of hyperglycemia on perinatal pulmonary cell turnover changes.

6.5 Neonatal rat lungs after exposure to maternal hyperglycemia and postnatal hyperoxia

In order to mimic the clinical situation and therapeutic intervention in newborns of diabetic mothers, we explored the notion of postnatal hyperoxic exposure as a second hit to the hyperglycemia-primed neonatal lungs. We considered a newborn rat with lungs in a saccular stage as an effective target to model the lung condition of a premature infant of diabetic mother (Burri, 2006). Our hypothesis was that the prenatal hyperglycemic exposure would sensitize the lung and therefore amplify the hyperoxia-induced lung injury. Corresponding to previous studies, hyperoxic exposure alone led to alveolar simplification with large, thin-walled terminal airspaces and decreased septation (Bourbon et al., 2005; Saugstad, 2010). In contrast to the separate insults of hyperglycemia or hyperoxia, the combined exposure attenuated the hyperoxia-induced impairment in alveolarization because the alveolar size remained at the level of control pups and this indicated that the primary septation was not affected. This phenomena was found also in a similar rat model in which hyperglycemia-primed lungs showed a smaller increase in mean airspace size than the hyperoxia control lungs (Sosenko and Frank, 1986). It is obvious that this finding was not due to increase in antioxidative capacity of the lungs because no difference was found in the antioxidative stress markers in both our study or in a previous study (Sosenko and Frank, 1986). Alike, in our study, no preventive effect on the survival of the pups after the dual exposure was found.

The combined exposure of hyperglycemia and hyperoxia prevented the hyperglycemia-induced septal wall thinning and decreased the number of secondary crests and pulmonary vessels simultaneously with increased apoptosis and diminished proliferation. This may be due to insufficient clearance or differentiation of the lipid interstitial cells that we found after the dual exposure but not in control lungs or after separate insults. These cells are normally present during the perinatal period and become highly apoptotic over the first 1-2 postnatal weeks in rats (Awonusonu et al., 1999) during the process of

alveolar thinning. The cells may differentiate to myofibroblasts, which have a central role in alveolar crest formation and disrupted transdifferentiation, on the other hand, may result in decreased number of secondary crests seen in our model after the dual exposure. The number of small pulmonary vessels was decreased after the combined exposure of hyperglycemia and hyperoxia, strengthening our study hypothesis of the amplifying effects of postnatal hyperoxia on prenatal hyperglycemic exposure. Since alveologenesis and vasculogenesis are closely interactive processes (Shannon and Hyatt, 2004), and disruption of angiogenesis during this critical period may impair alveolarization and interface with the maintenance of alveolar structures (Stenmark and Abman, 2005; Thébaud and Abman, 2007), it may be speculated that the same mechanisms may contribute to both the inhibition of septal thinning and decrease in vascularization detected in this study.

7 SUMMARY AND CONCLUSIONS

Maternal hyperglycemia induced major gene expression changes in developing tissues. Maternal type 1 diabetes downregulated the blood vessel development - and extracellular matrix -related genes. This is in accordance with previous studies in which a diabetic environment induced structural changes in vascular and extracellular matrix regions in human umbilical cords and produced embryonal vasculopathy in animal tissues. Additionally, maternal diabetes induced expression changes in genes involved in the regulation of vascular tone. The expression profile favored vasodilatation, which may promote adequate blood flow to the fetus and be a reflection to the delayed postnatal cardiopulmonary adaptation frequently seen in newborns of diabetic mothers (I).

Maternal hyperglycemia had a prolonged influence on the gene expression profile in neonatal rat lungs during late alveolarization. The gene expression of extracellular matrix proteins was upregulated, which reflected perturbations in extracellular matrix modeling. Cellular proliferation - and antioxidant-related genes were downregulated, concomitantly. This may reflect a compensatory change during recovery after intrauterine exposure and these alterations may either be permanent aberrations, temporary reactions or attempts to balance lung growth. This prolonged downregulation of antioxidant genes together with the altered regulation of extracellular matrix and cellular proliferation may predispose the lungs to further postnatal insults (II).

Maternal hyperglycemia affected the neonatal rat lung structural development. The lungs showed premature remodelling from birth to the late alveolarization. At birth, the saccular walls were thin, the lung total tissue area and relative weight were decreased, and apoptosis and proliferation were increased. This indicates premature development and enhanced cellular turnover, which may lead to deviated lung growth. Postnatally, the lungs showed prolonged pulmonary remodelling, since septa remained thinner up to the age of one week, the number of secondary crests further increased at two week of age and cell proliferation was stimulated during the whole alveolarization period. Together with our gene expression data, this may reflect a persisting effect of intrauterine hyperglycemia on postnatal lung growth control mechanisms (III).

Corresponding to previous studies, hyperoxic exposure alone led to alveolar simplification with large, thin-walled terminal airspaces and decreased septation. In contrast, the combined exposure of maternal hyperglycemia and postnatal hyperoxia did not affect the alveolar size but the number of secondary crests was decreased and the pulmonary maturation showed an arrestment with thick alveolar walls with an abundance of lipid interstitial cells and a lack of small pulmonary vessels and caused decreased survival of the pups. Oxidative stress markers and antioxidants remained unchanged but alveolar wall apoptosis was increased and cell proliferation diminished highlighting an imbalance. The insufficient clearance or differentiation of the lipid interstitial cells found after the

dual exposure may explain the thick alveolar walls and the lack of secondary crests but warrant further investigation. The decreased number of small pulmonary vessels after the dual exposure confirmed our study hypothesis of the amplifying effects of postnatal hyperoxia on prenatal hyperglycemic exposure (IV).

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