Aus dem Zentrum für Kinder- und Jugendmedizin der Universität zu Köln

Klinik und Poliklinik für Kinder- und Jugendmedizin

Direktor: Universitätsprofessor Dr. med. J. Dötsch

Dysregulation of angiogenic signaling and reduced elastic fibers are associated with impaired formation of microvessels in rat lungs after intrauterine growth retardation.

Inaugural-Dissertation zur Erlangung der Doktorwürde

der Medizinischen Fakultät

der Universität zu Köln

vorgelegt von

Centina Kuiper-Makris

aus Tiel (Niederlande)

Promoviert am 28. Januar 2022

Aus dem Zentrum für Kinder- und Jugendmedizin der Universität zu Köln

Klinik und Poliklinik für Kinder- und Jugendmedizin

Direktor: Universitätsprofessor Dr. med. J. Dötsch

Dysregulation of angiogenic signaling and reduced elastic fibers are associated with impaired formation of microvessels in rat lungs after intrauterine growth retardation.

Inaugural-Dissertation zur Erlangung der Doktorwürde

der Medizinischen Fakultät

der Universität zu Köln

vorgelegt von

Centina Kuiper-Makris

aus Tiel (Niederlande)

Promoviert am 28. Januar 2022

Gedruckt mit Genehmigung der Medizischen Fakultät der Universität zu Köln
Druckjahr: 2022

Dekan: Universitätsprofessor Dr. med. G. R. Fink
1. Gutachter: Juniorprofessor Dr. med. Dr. nat. med. M. A. A. Alcazar
2. Gutachter: Universitätsprofessor Dr. rer. nat. B. Brachvogel

ERKLÄRUNG

Ich erkläre hiermit, dass ich die vorliegende Dissertationsschrift ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskriptes habe ich Unterstützungsleistungen von folgenden Personen erhalten:

Jun. Prof. Dr. med. Dr. nat. med. M. A. A. Alcazar.

Weitere Personen waren an der geistigen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe einer Promotionsberaterin/eines Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertationsschrift stehen.

Die Dissertationsschrift wurde von mir bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Erklärung zur guten wissenschaftlichen Praxis:
Ich erkläre hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten (Amtliche Mitteilung der Universität zu Köln AM 24/2011) der Universität zu Köln gelesen habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen.

Köln, den 16.03.2021 Unterschrift:

ERKLÄRUNG

Die dieser Arbeit zugrundeliegenden Experimente sind von mir mit Unterstützung von der Biologielaborantin Frau C. Vohlen durchgeführt worden. Der Anteil der verschiedenen Ko-Autoren an der Publikation “Mendelian randomization and experimental IUGR reveal the adverse effect of low birth weight on lung structure and function”(1) ist in Kapitel 3 (contribution of coauthors, Seite 35) aufgelistet.

Köln, den 16.03.2021 Unterschrift:

INDEX

LIST OF ABBREVIATIONS

4E-BP1	Eukaryotic Translation Initiation Factor 4E-binding Protein 1
AEC I	Alveolar Epithelial Cell Type I
AEC II	Alveolar Epithelial Cell Type II
ALK	Activin Receptor-like Kinases
AMPK	AMP-activated Serine/Threonine Protein Kinase
Ang1	Angiopoetin 1
Ang2	Angiopoetin 2
Apln	Apelin
BMP	Bone morphogenetic protein
BPD	Bronchopulmonary Dysplasia
CD31	Cluster of Differentiation 31
Cdh5	Cadherin 5, vascular endothelial Cadherin
CO_2	Carbon dioxide
COPD	Chronic Obstructive Pulmonary Diseases
CPAP	Continuous Positive Airway Pressure
ECM	Extracellular Matrix
ELBW	Extremely Low Birth Weight (<1000g)
Eln	Elastin
Erk1/2	Extracellular Signal Regulated Kinases 1 and 2 (Erk42/44)
FEV1	Forced Expiratory Volume per second
Flt1	Feline McDonough Sarcoma (FMS) related receptor tyrosine kinase 1
Flk1	Fetal liver kinase 1
HIF	Hypoxia Inducible Factor
ID-1	Inhibitor of DNA-binding protein 1
IGF-1	Insulin-like Growth Factor-1
IUGR	Intrauterine Growth Restriction
KLF4	Krüppel-like Factor 4
LBW	Low Birth Weight (<2500g)
MAP	Mitogen-activated Protein
MD	Mendelian Randomization
MMP	Metalloproteinase
mTOR	Mechanistic Target of Rapamycin
nCLD	neonatal Chronic Lung Diseases
O_2	Oxygen
PAH	Pulmonary Arterial Hypertension
PDGF-A	Platelet Derived Growth Factor A
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PPHN	Persistent Pulmonary Hypertension of the Newborn
SD	Standard Deviation
SGA	Small for Gestational Age
SMAD	SMA (small worm type) and MAD (Mothers against Decapentaplegic genes)
SP	Surfactant Protein
TGFβ	Transforming Growth Factor β
Tie2	Tyrosine kinase with immunoglobulin-like and EGF-like domains 2
VE-Cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VLBW	Very low birth weight (<1500g)
vWF	von Willebrand Factor
Wnt	Wingless-type family

1. INTRODUCTION

1.1. The lung

1.1.1. Lung structure and function

The lung is our respiratory organ, its function is vital for our survival from the minute we are born. The structure of the lung is designed to provide the largest surface possible for the exchange of oxygen and carbon dioxide. Oxygen (O_2) is absorbed from the air we breathe and transported from the lungs to all the tissues in the body. There, it is indispensable for the production of energy and other cellular functions. The byproduct of these processes is carbon dioxide (CO_2), which is toxic for the body and needs to be transported back to the lungs, where it can be cleared (2-4). In consequence, the lung has to be able to absorb as well as transport gases. In order to establish this process in the most efficient way, it needs to fulfill two characteristics: (1) the largest surface possible for gas exchange, and (2) the thinnest barrier possible between air and blood (5). When inhaling, the air is transported into the lung via the conducting airways: trachea, bronchi and bronchioli, in decreasing diameter (see figure 1, provided by the LUNGevity foundation). In the peripheral lung areas, millions of small air sacs, or 'alveoli', serve as the optimal environment to absorb oxygen from the air. The oxygen molecules are bound and transported through the thin alveolar wall into the capillaries that make up the lung vascular bed. In the opposite direction, CO_2 is transported from the blood into the alveoli. Through exhaling, the CO_2 enriched air is forced out of the alveoli and back up into the conducting airways (6). This process continues in a loop of in- and exhaling our entire lives.

This work focusses on the development of the peripheral lung tissue; the alveoli and the millions of capillaries (blood vessels, <100 μm) that align the alveolar walls. The alveolar walls, or ´epithelium´, consist of two types of cells: the alveolar epithelial cell 1 and alveolar epithelial cell 2 (AEC I and AEC II, respectively). The AEC I are thin cells that spread out with a minimal thickness of ca. 25 nm (7). They cover approximately 95% of the alveolar surface. The remaining 5% are AEC II,

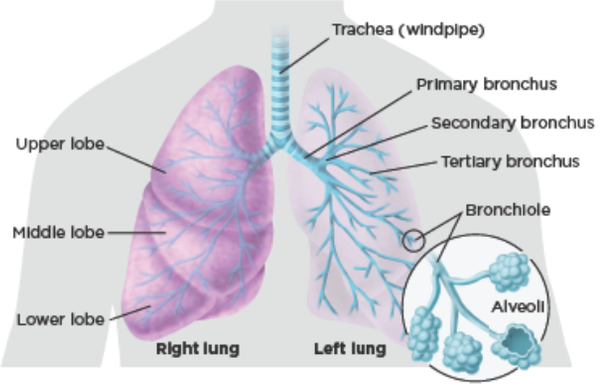

Figure 1: lung anatomy, depicting the conductive airways and the alveoli. © LUNGevity foundation, copyright permission granted.

their main function is to produce surfactant proteins (SP)(7, 8). Surfactant is a thin fatty film that lies over the alveolar wall in order to reduce the surface tension and to provide maximum unfolding of the alveoli (8). In addition, AEC II have the ability for self-renewal and can differentiate into other cell types. The AEC II are therefore considered to be alveolar progenitor cells. These abilities are illustrated by the AEC II giving rise to AEC I after lung injury, thereby contributing to alveolar regeneration (9).

1.1.2. Lung development

Pulmonary development is divided into five phases: the **embryonic**, **pseudoglandular**, **canalicular**, **saccular** and **alveolar** phase (figure 2). The alveolar phase and the subsequent microvascular maturation continues after birth and beyond infancy (10, 11). For the purposes of this study, a further understanding of the processes taking place during these different stages is essential for a grasp on the consequences of adverse prenatal influences such as growth restriction or prematurity. As will become clear, the last two stages of development are closely related to the respiratory capacity at birth. In the following, the five stages of lung development will be briefly described.

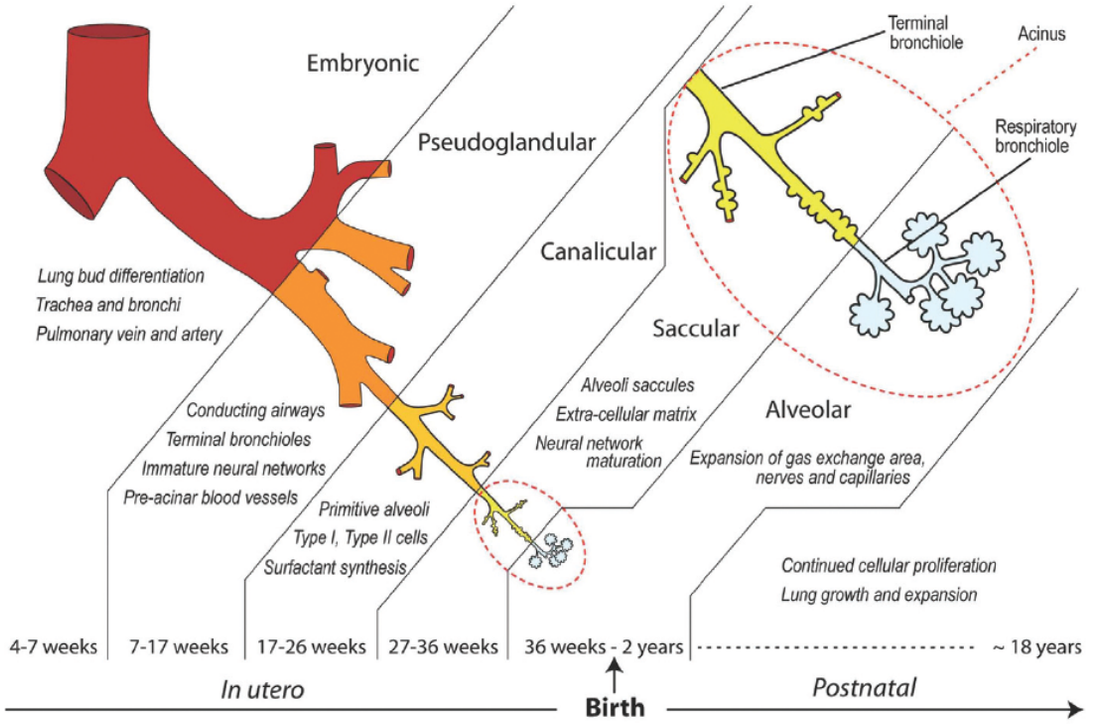

Figure 2: stages of human lung development, before and after birth. Source: Kajekar R, Pharmacol Therap 114, 2007, DOI: 10.1016/j.pharmthera.2007.01.011 (12). Reprinted with permission from Elsevier, License number 4972971030423.

During the **embryonic phase**, which spans from the 4th to 7th week of pregnancy in humans, the lung cell progenitors develop from the foregut endoderm, initially called the lung buds. From there, epithelial and vascular structures arise via branching morphogenesis. This development is regulated by Wnt (Wingless-type family) and BMP

(Bone Morphogenetic Protein) signaling, amongst others (13, 14). Simultaneously, the mesoderm gives rise to all supporting structures (e.g. connective tissue, smooth muscle, lymphatics and pleura) as well as endothelial cell precursors . With the start of the **pseudoglandular phase** (gestational weeks 5-17), the lung buds develop from the endodermal tissue into the mesoderm and subsequently develop a tubular formation that will mature into the conducting airways (15-17). During the **canalicular phase** (gestational weeks 16-26), the airway epithelium further proliferates and differentiates into conductive and future respiratory surface. In addition, the first structural organization of future blood-air barriers occur and the AEC II start the production of surfactant (18, 19). Next is the **saccular phase** (gestational weeks 24-38), in which some of the last minor branching morphogenesis take place, before **alveolarisation** starts (19).

At the end of the airspaces, wider sacs are formed, and where these sacs meet, arise thick primary septae (19). These primary septae consist of a central layer of connective tissue between two capillary sheets (20, 21). During the alveolar phase (gestational week 36 until early adulthood (11)), secondary septae grow from the primary septum into the open (future) airspace (figure 3). Secondary septae are thinner and consist of a double layer of fusing capillaries and only little connective tissue, and are covered by the thin AEC I´s (22). The direction of growth of secondary septae appears to be mainly controlled by cell-cell and cell-matrix interaction: the collection of elastic and collagen fibers by progenitor smooth muscle cells at the future tip of the septum (23-25), and the concurrent outgrowth of capillaries (26, 27).

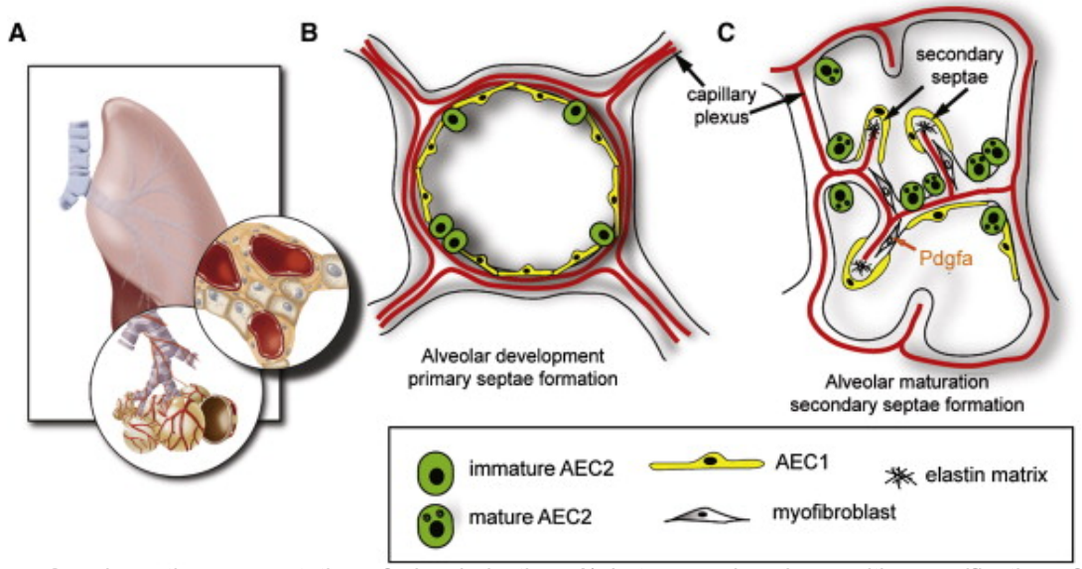

Figure 3: schematic representation of alveolarisation. A) Late saccular phase with magnification of the terminal bronchiole and attached saccules. B) Development of primary septae, with emergence of AEC I (AEC1) and AEC II (AEC2) cells. C) Formation of secondary septae and start of surfactant production by the AEC II´s. Morrisey and Hogan, 2009 (28), reprinted with permission of Elsevier, license number 4994000701767.

Every step of airway formation and alveologenesis is accompanied by development, outgrowth, migration and maturation of the pulmonary vascular bed. A concert interaction of growth factors (discussed in detail in chapter 1.3.), with vascular endothelial growth factor (VEGF) in its center, orchestrates the complex process of vasculogenesis and subsequent angiogenesis. During the **embryonic phase**, blood vessels originate by means of vasculogenesis from differentiated mesenchymal tissue called hemangioblasts, forming into blood lakes in the tissue surrounding the lung buds (17, 29). With the start of the **pseudoglandular phase**, mesenchymal tissue becomes apoptotic, decreasing the space between endothelium and airway epithelium (17). In addition, a capillary plexus forms around the developing airways by massive migration of endothelial cells, which forms a secondary connection to the main circulation (30). The **canalicular phase** is characterized by a further lining of the airways by blood vessels in response to VEGF, expressed by the differentiating epithelial cells (31). The **saccular** and **alveolar phases** are furthermore characterized by a diminishing of the space between epithelium and endothelium as well as further growth of the capillary bed under increasing expression of VEGF concentrations (32). The 'sprouting of blood vessels from pre-existing vessels' is termed angiogenesis (33, 34). Angiogenesis, however, is not the only method of growth. Parallel to this process occurs intussusceptive microvascular growth, during which a sheet of connective tissue divides the lumen of one capillary into two (35).

The capillary network keeps developing after finalization of alveolarisation and is completed in a process called 'microvascular maturation'. It spans from several months after birth until adolescence (11). In this phase, the two layers of capillaries within the secondary septae fuse to form a single capillary sheet (36). After microvascular maturation, changes in the number of capillaries are still possible through microvascular remodeling. This remodeling allows for a continuous growing and regression of vessels under the influence of environmental factors (e.g. ischemia, inflammation, wound healing) and a persistent potential for lung growth into adulthood (25, 37, 38).

The close relationship between angiogenesis and alveolarisation has been studied in detail (39-43). It has been shown that impaired angiogenesis through inhibition of angiogenic factors such as VEGF or angiopoetins leads to arrest of alveolarisation (27, 39). As epithelial cells express VEGF to increase proliferation of endothelial cells, these cells in turn express hepatocyte growth factor (HGF) to induce the formation of primary septae in return (44). The same reciprocal interaction between the two cell types can be observed in the formation of secondary septae. The accumulation of elastin in specific

areas of the primary septae signals the location of outgrowth of the secondary septae. Elastin attracts endothelial cells and induces angiogenesis into the secondary septum (45). In return, the endothelial cells produce retinoic acid, which increases the synthesis and deposition of elastin (46).

Pulmonary development, including alveolarisation, is tightly orchestrated by a large set of growth factors that also control accompanying angiogenesis and proper extracellular matrix (ECM) formation. A complex interaction between all these factors is the fundamental basis for proper lung growth and function. Dysregulation of these factors, i.e. an imbalance of growth factors, can disrupt alveolarisation and lead to aberrant lung growth and ultimately lung disease. Critical adverse influences comprise antenatal and postnatal factors (such as mechanical ventilation and hyperoxia)(47-53). Antenatal factors include **maternal** (e.g. preeclampsia (54), inflammation (55), malnutrition (56), obesity (57)), **placental** (placental insufficiency due to preeclampsia (58)), and **fetal** factors (e.g. genetics (59)). These factors can cause significant adverse changes to the lungs, but also induce the risk of a second hit to the developing fetus: premature birth.

1.2. The lung at birth

The most drastic pulmonary transformation takes place at birth. In the intrauterine environment, the lung is filled with amniotic fluid and fetal lung fluid that allow for a positive pressure within the lungs, pushing the alveoli to unfold and expand (60, 61). During and after birth, the fluid is in part removed from the lungs by mechanical forces, and in part resorbed by the lungs, and replaced by air (60). When the lung is ‘dry’, the surfactant that has been produced in the last weeks before birth by the AEC II´s is essential to decrease the surface tension within the alveoli and allow for their proper expansion (8). This process is usually completed within hours from birth.

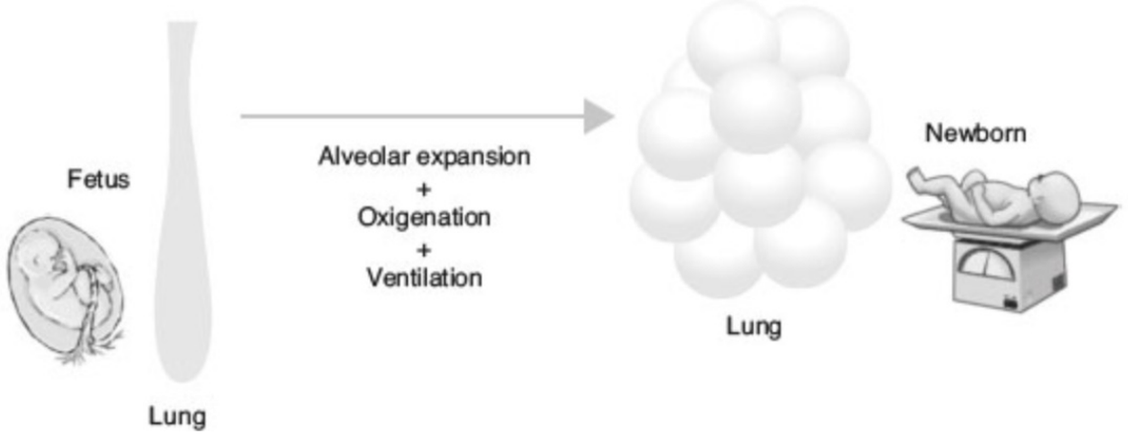

Figure 4: schematic of the pulmonary transition at birth: from a fluid filled state, to full expansion and air-filled alveoli. Altered picture after Cabral and Belik, 2013 (62), reprinted with permission from Sociedade Brasileira de Pediatria [Brazilian Society of Pediatrics].

1.2.1. Prematurity

Infants born before finalizing 37 weeks of pregnancy are born premature (63). Prematurity is related to a high risk of postnatal complications, due to the immaturity of all organs. These complications include intracerebral or intraventricular hemorrhage, persisting ductus arteriosus, necrotizing enterocolitis, high rates of infection and feeding intolerance (64, 65). In addition, their lungs are not fully developed; alveolarisation is incomplete, the vascular bed is still in the early stage of maturation and the surfactant production is not yet sufficient. Consequently, pulmonary complications have a high incidence in premature neonates (65). The respiratory distress often requires supportive oxygen therapy and mechanical ventilation/continuous positive airway pressure (CPAP)(52). While these treatments are indispensable in the majority of the cases, they also increase the risk for neonatal chronic lung disease or bronchopulmonary dysplasia (51, 53) with life-long pulmonary sequelae. As epidemiological studies have pointed out, premature infants have a higher risk for chronic obstructive pulmonary diseases (COPD)(66), emphysema (67, 68), asthma (69-71) and pulmonary arterial hypertension (72, 73).

1.2.2. Bronchopulmonary dysplasia

A major pulmonary complication of premature birth is bronchopulmonary dysplasia (BPD). The ‘old BPD’, initially described by Northway et al. in 1967, was referred to as a need for supplemental oxygen at postnatal day 28 as well as abnormal radiological findings in the lungs, that include ´a cystlike appearance´ and signs of membranous disease (74, 75). Advances in neonatal management over the last decades, including surfactant therapy and antenatal corticosteroids along with the avoidance of mechanical ventilation and high oxygen supplementation, have improved the treatment of postnatal respiratory distress and increased the survival rate of preterm neonates considerably. The changes in neonatal management have led to a transformation of the ‘old BPD’ into the ´new BPD’ (76). New BPD, also called neonatal chronic lung disease (CLD), is characterized by the preterm neonate’s need for supplemental oxygen therapy at 36 weeks of corrected age (77, 78). The German society of neonatology and pediatric intensive care medicine (GNPI) has defined grades of severity for BPD, measured at gestational week 36 and/or discharge from the hospital; patients with mild BPD are respiratory stable with room-air (21% O_2), patients with moderate BPD require little additional oxygen (22-29% O_2) and patients with severe BPD require high concentrations of additional oxygen (>30% O_2 and/or CPAP/ventilation) (GNPI *Leitlinie*, *Register-Nummer*: 024-014, Klasse: S2, Stand: 01.06.2009). Histologically, BPD lungs are characterized by an arrest of pulmonary development with a lack of alveolarisation and

microvasculature (47, 50, 76, 79). In severe cases of BPD, the lungs also show structural damage with large areas of emphysema and fibrosis (80).

BPD is associated with prolonged postnatal hospitalization and mortality. In addition, infants with BPD have an increased risk of childhood diseases (e.g. growth failure, cardiovascular diseases, neurodevelopmental disorders) when compared to premature children without BPD (81). The incidence of BPD varies per group of premature infants; the incidence is much higher in the extremely premature and extreme-low-birthweight (ELBW, <1000g) infants. In addition, the rates differ largely between the different definitions of BPD and between institutions. In Europe, the incidence of BPD was 10,5-21,5% for infants born before 32 weeks of gestation in 2003 (82), and in the US ca. 50% for premature infants born with an extremely low birth weight (<1000g) (65). Between 2006 and 2017, the global incidence rate for BPD was 17-75%, dependent on the gestational age, birthweight and the amount of medical care available (83).

As stated before, prematurity causes respiratory distress, requiring supportive oxygen therapy and ventilation, thereby causing lung injury and inducing BPD (51-53, 65). However, not all premature infants develop BPD, because additional risk factors can determine the susceptibility for BPD (77). Amongst others, these additional risk factors for BPD comprise IUGR, pre-eclampsia and prenatal inflammation (chorioamnionitis)(81). In addition, BPD in itself is a risk factor for the development of other pulmonary diseases as well; multiple studies have shown that children with a history of BPD have long-term impaired lung function as well as an increased risk of asthma (66, 84, 85). Adolescents and adults born premature are prone to develop asthma, COPD or pulmonary hypertension as well (66, 71, 84, 86). This clearly shows the long-term consequences of premature birth on lung development and highlights the importance of adequate therapy and prevention of (respiratory) complications, for which knowledge about the pathophysiology is essential.

1.3. Intrauterine growth restriction (IUGR)

Intrauterine growth restriction (IUGR) indicates an abnormal low birth weight for the gestational age (87, 88). It was originally assumed that infants with a low birth weight were all premature and the gestational age of the infant had been wrongly calculated. However, in the 1950's and 1960's, the first reports of a distinction between prematurity and IUGR arose (89, 90). Classically, IUGR was defined as a birthweight below 2500 grams (87). More recently however, it has been characterized as 'not reaching the biologically based potential', which accounts for a clinical diagnosis at birth, as well as

close monitoring of the intrauterine growth (88, 91). Consequently, infants that show evidence of underperfusion *in utero* or malnutrition at birth are diagnosed with IUGR (92).

IUGR is often confused with 'small for gestational age' (SGA), which indicates a birthweight of -2 SD/mean and is therefore an exact measure (93). However, IUGR can occur in infants that have a normal birthweight, just as an infant with SGA might not have IUGR (92). Over the past decades, many epidemiological studies have been performed to assess the effects of IUGR. However, due to changing definitions, not all studies have used the same measures for IUGR. In addition, most recent studies interchange IUGR and SGA as synonyms, which makes it increasingly difficult to interpret and compare studies.

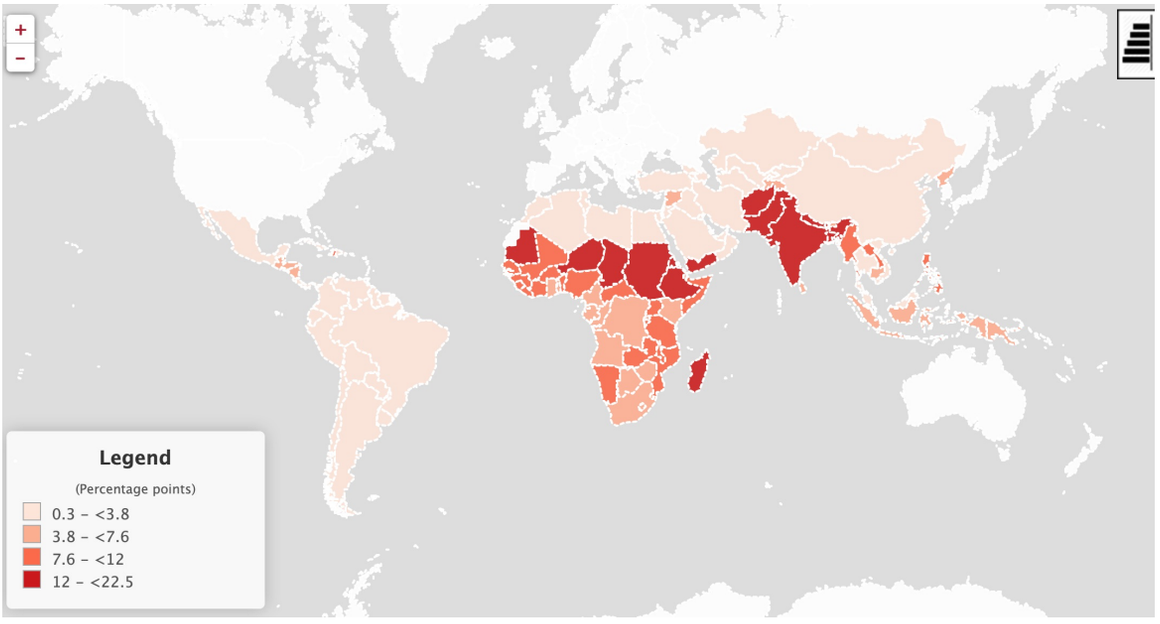

Figure 5: low birth weight (cut off: <2500g) prevalence in developing countries in 2011(94). © 2016 Danaei et al., distributed under the terms of the Creative Commons Attribution License.

IUGR affects approximately 10% of all liveborns, with up to six times higher incidences in developing countries (figure 5)(94, 95). IUGR often coincides with prematurity; about 30-50% of all extremely premature infants are IUGR (96). The causes for IUGR can be divided into two major groups: maternal factors (including placental factors) and fetal factors. **Maternal factors** include maternal comorbidity (e.g. diabetes, preeclampsia (97)), malnutrition (98) and deficient utero-placental function (99). The second group includes all **fetal factors**, e.g. congenital disorders (100) or intrauterine infections (101). All infants with IUGR tend towards a post-natal catch-up growth during the first 2 years of life, if the proper treatment of perinatal complications and appropriate nutrient intake can be provided (102).

1.3.1. IUGR and postnatal pulmonary complications

Independent of prematurity, IUGR infants are at an increased risk for perinatal asphyxia (associated with meconium aspiration syndrome) and neonatal (adaptation-) complications, resulting in an increased mortality rate of 10-20 times (96, 103). In the early postnatal period, there is a significantly increased risk of pulmonary complications such as persistent pulmonary hypertension of the neonate (PPHN)(104), respiratory distress syndrome (105) and pulmonary hemorrhage (106). In addition to these acute respiratory complications, IUGR infants are also at increased risk for BPD (107). Since IUGR generally occurs in the last stages of pregnancy, during the late saccular and alveolar phase of development, the changes might be comparable to those seen in premature born infants. Indeed, animal models for IUGR have shown a lack of alveolarisation and an increase of ECM deposition; these changes are similar to those seen in lungs of premature newborns and infants with BPD (108-111). As explained in chapter 1.1.2., angiogenesis plays a vital role in alveolarisation. The question therefore arises whether the lack of alveolarisation is the result of dysregulated angiogenesis.

1.3.2. IUGR causes perinatal programming

In the late 20th century, David Barker was the first to hypothesize a causal relationship between IUGR, low birth weight and prematurity, and adults' diseases such as arterial hypertension, coronary heart disease and type 2 diabetes (112, 113). This hypothesis was supported by the observations of large cohort studies that clearly reflect this relationship (114-120). It became known as the 'thrifty phenotype hypothesis', which assumes a fetal programming in the late intrauterine phase in response to its environment and the consequences of which persist throughout life (121). From an evolutionary viewpoint, this results in a genetic alteration of the individual that allows for a better adaptation to an environment with deficient nutrient supply. If, however this depleted environment does not persist, the adapted individual cannot metabolically cope with the nutritional richness and is more likely to have a pathologic accumulation of these nutritional extras (122-125). The result is a mismatch of prenatal restricted growth and postnatal accelerated growth, due to a mismatch of fetal adaptation and environmental exposure (126, 127). Epidemiological studies have shown that IUGR and consecutive catch-up growth cause an increased risk for early childhood obesity and metabolic diseases such as diabetes, metabolic syndrome, cardiovascular diseases, hypertension and chronic kidney diseases (114, 115, 119, 128-130). In contrast, experimental models of IUGR have shown that a postnatal restriction of nutritional intake decreases the risk for metabolic disease (131, 132).

1.3.3. IUGR and chronic lung diseases

IUGR and the therewith associated catch-up growth are major risk factors for childhood pulmonary disease. Recent studies in experimental IUGR and infants born IUGR have shown an increased risk of morbidity in all organ systems, but especially the lung, later on in life (74, 133, 134). Several cohort studies have shown that schoolchildren born IUGR have a significantly lower FEV1 and airway resistance as well as a higher susceptibility to airway infections, independent of catch-up growth (70, 71, 135, 136). Moreover, in long-term follow-up studies it was shown that a low birthweight (the old definition of IUGR) decreases lung function in adulthood, with a reduction of lung capacity and elasticity, resembling a COPD phenotype (56, 137).

The postnatal catch-up phase overlaps with the final stages of pulmonary alveolarisation and vascular maturation (138). Previous research has shown that the influence of catch-up growth on lung development is most notable in the organization of the ECM (108, 133, 139). In addition, accelerated postnatal weight gain is clinically associated with early-childhood obesity and pulmonary symptoms such as asthma and wheezing (140). It becomes clear that the influence of IUGR on the lung has two phases: altered lung development, followed by altered lung maturation. In other words, the two-hit model of the Barker-hypothesis, of prenatal intrauterine growth restriction and postnatal extrauterine growth acceleration applies to the lung as well.

1.4. Angiogenic signaling

The growth and differentiation of all organs depend on the specific spatio-temporal expression of growth factors as well as interactions between the different cell types and components of the developing tissues. The individual cells in the developing tissue receive signals from outside the cell (amongst which are transcription factors and growth factors) that determine the cell fate (141). The location and concentration of these factors are guiding the cell. Accordingly, the precise differentiation and spatial organization of the blood vessels is directed by an intricate process comprising many growth factors along with intercellular communication (33, 142).

1.4.1. Angiogenic signaling: the vascular endothelial growth factor (VEGF) pathway

Vascular endothelial growth factor (VEGF) is the best-studied angiogenic factor. There are several subtypes of VEGF (in humans VEGF-A to VEGF-D) and three receptors (VEGFR1, VEGFR2 and VEGFR3)(143). The most significant role in angiogenesis is

reserved for VEGF-A, which exerts its function through VEGF Receptor 1 (VEGFR1, also known as Flt-1) and VEGFR2 (also known as Flk-1), both expressed on endothelial cells (144). VEGFR2 is an early molecular indicator of the endothelial cell precursor (145). In addition, studies with recombinant human Recombinant Human $VEGF^{165}$ have shown that it also directly binds to alveolar type II cells, stimulating proliferation and differentiation (146). The expression of VEGFs is induced by several stimuli, and exerts its influence on the proliferation, migration and differentiation of endothelial cells (26, 147-149) through several signaling pathways, influenced by a vast body of pro- and antiangiogenic factors. The following segment provides an overview of the current knowledge on the role of VEGF in pulmonary development.

Hypoxia is a well-documented stimulator of sprouting activity during tissue development (150-154). An essential mediator in this process are the hypoxia inducible factors (HIF). Under normoxic circumstances, HIF proteins are degraded by signaling of prolyl hydroxylase domain (PHD) proteins, that function as oxygen sensors (155). Under hypoxia, these proteins do not hyroxylate HIF, allowing them to induce the transcription of genes promoting direct oxygen flow to the tissue, while also stimulating angiogenesis (152, 155). An important part of pro-angiogenic action is their binding to the VEGF gene promotor (150). Early exposure of the developing lung to hyperoxia appears to downregulate HIF and VEGF expression (156), a mechanism that could occur in premature birth and respiratory therapy, and lead to BPD (157). This process is based on a chemoattractive gradient, in which the growth factor is expressed from a central source and diffuses over the tissue, directing growth in the direction of the source (158). The direct inhibition of angiogenesis *in vivo* with VEGF-inhibitors causes a BPD-like phenotype with reduced alveolarisation (39, 159). Reversely, animal studies have shown that this lack of alveolarisation induced by inhibition of angiogenesis can be partly rescued when angiogenesis is induced by addition of angiogenesis stimulating factors such as nitric oxide and retinoic acid (46, 159). These studies highlight the importance of VEGF for proper lung development, and its sensitivity to other signaling molecules.

VEGF is one of the growth factors that activates the converging pathway of extracellular signal regulated kinases 1 and 2 (Erk1/2 or Erk42/44)(160-163). ERK1/2 is a member of the mitogen-activated protein (MAP) kinases and part of the larger Ras-Raf-MEK-ERK signal transduction cascade, which has a central function in tissue development and the maintenance of various cell functions such as survival, differentiation and migration (figure 6)(46, 162, 164). In addition to VEGF, other factors like cytokines, epidermal growth factor, insulin and insulin-like growth factor, platelet-derived growth factor (PDGF)

as well as osmotic stress also activate ERK1/2 (165, 166). VEGF induces Erk1/2-mediated promotion of epithelial cell survival (167), as well as migration, proliferation and differentiation of endothelial cells (160).

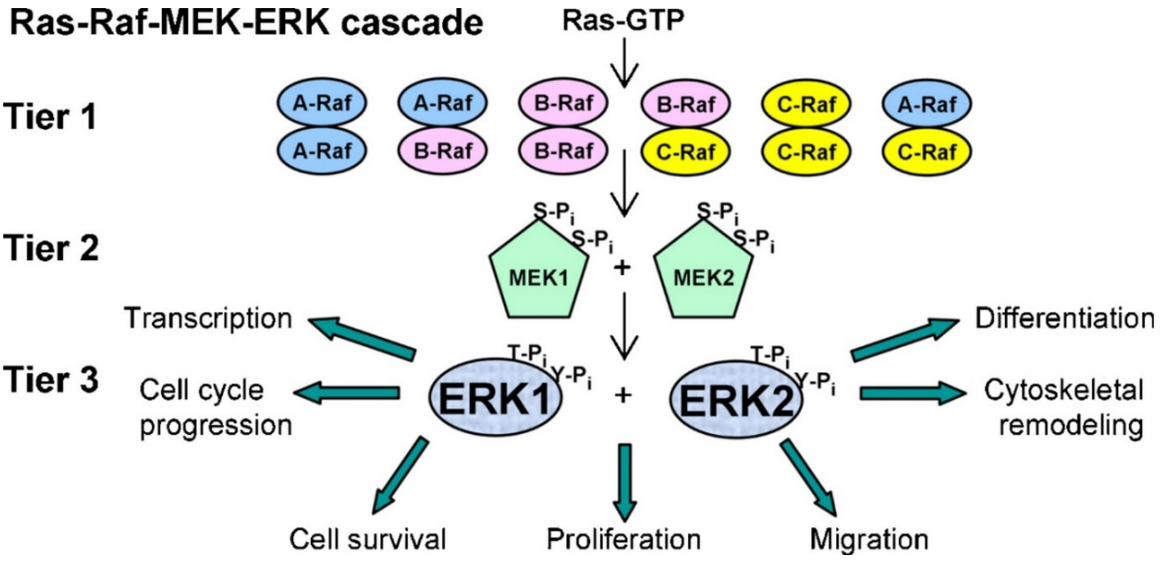

Figure 6: The Erk1/2 serine/threonine kinases are part of the Ras-Raf-MEK-ERK signal transduction cascade that regulates various vital cellular processes. The signaling cascade is activated by growth factors such as VEGF that activate Ras-GDP into Ras-GTP, setting the cascade into motion by the activation of the Raf kinase family (A-Raf, B-Raf and C-Raf) that subsequently phosphorylate MEK1/2 (Mitogen-activated protein kinase). Roskoski Jr., 2012 (166), reprinted with permission of Elsevier, license number 4972991146840.

1.4.2. Angiogenic signaling: the bone morphogenetic protein (BMP) pathway

Another group of growth factors that regulates pulmonary angiogenesis is the bone morphogenetic protein (BMP) family. Originally, the BMP family was investigated in the field of bone and cartilage research. However, subsequent studies identified diverse functions of the BMPs in various tissues ranging from organ development to maintenance of vascular homeostasis (amongst others)(168). In particular, BMP receptor 2 (BMPR-II) is critical in the pathogenesis of pulmonary arterial hypertension (PAH); about 20% of the patients with idiopathic PAH and more than 70% of patients with familiar PAH exhibit mutations in the *Bmpr2* gene (169-171). Indeed, a heterozygous, lung-specific BMPR-II knockout induces a PAH phenotype in a mouse model (172). Moreover, an increased activity of the BMP-signaling pathway has been described at atherosclerotic sites, indicating a stiffening of the extracellular matrix (ECM)(173). In more recent years, the BMP family has raised interest in cancer research, due to its role in angiogenesis. It has been shown that BMP directly influences neovascularization during tumor growth (174). Recent studies, in addition, have also indicated a role for BMP in the response to nutritional changes. It has been shown for example, that activation of bile acid signaling after food uptake, in part due to glucagon-

like peptide-1 (GLP-1), activates BMP4 expression (175, 176). This pluripotent family of growth factors therefore deserves further studying.

The BMPs are members of the Transforming Growth Factor-β (TGFβ) family, encompassing over 20 molecules (177). The BMPS bind to two groups of receptors: BMP type I (activin receptor-like kinases 1-7 (ALK-1-7), including BMPR-IA and BMPR-IB) and type II receptors (BMPR-II)(177). For pulmonary development and angiogenesis, BMP2/4/9/10, BMPR-IA, BMPR-IB and BMPR-II are of interest. Because there is such a variety of BMPs working in concert, the present study primarily focused on the BMP receptors. The type I receptors mainly activate the signaling cascade of Smad1, 5 and 8 (figure 7)(177). Interestingly, a crosstalk with the VEGF signal transduction of Erk1/2 has been observed here: Erk1/2-signaling is a regulator for BMP-4 dependent capillary sprouting (178).

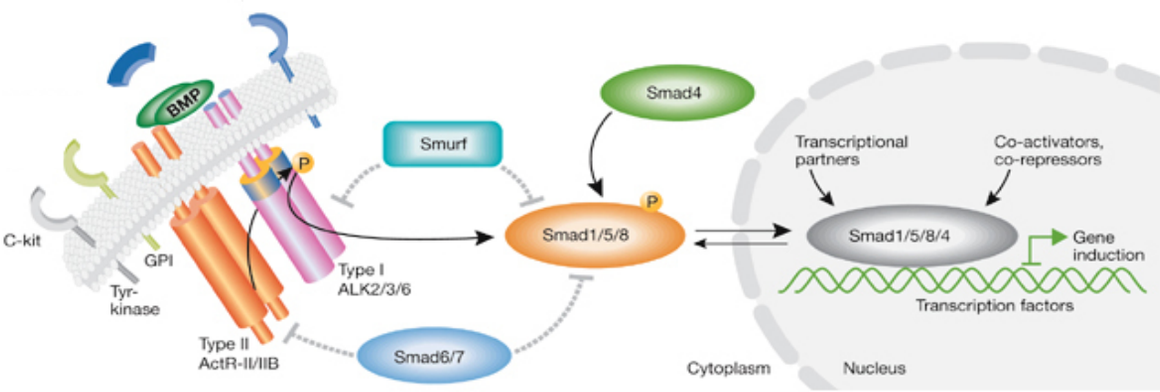

Figure 7: SMAD-dependent bone morphogenetic protein (BMP) signaling. BMPs induce heterodimeric complex formation between two BMP receptors (BMPRs). The type II receptor kinase phosphorylates the type I receptor and subsequently activates intracellular signaling. Upon BMPR activation, Smad1–Smad5–Smad8 forms heterodimeric complexes with Smad4, which then translocate to the nucleus where they act directly and/or cooperate with other molecules to regulate the transcription of target genes. Inhibitory Smad6–Smad7 specifically inhibits BMP signaling. Simic and Vukicevic, 2007 (179). Reprinted with permission of Wiley and Sons, license number 4998120131556.

The activation of the BMP receptors induces the expression of many effector genes. Worth noticing in the light of pulmonary angiogenesis are apelin, inhibitor of DNA-binding protein 1 (ID1) and Krüppel-like factor 4 (Klf4). BMP reduces apelin expression under hypoxic circumstances, thereby inhibiting hypoxia-induced endothelial cell proliferation (180, 181). Apelin agonism has even been demonstrated to be an effective treatment for PAH by promoting angiogenesis via sprouting *in vitro* as well as in rodent models and in humans (182-185). In addition, treatment of hyperoxia-induced BPD with apelin agonists has been shown to increase alveolarisation in a rat model (186). The transcription factors ID-1 and Klf4 represent another crosstalk between the BMP and VEGF-signaling pathway. ID-1 is an activator and effector gene of both the VEGF-Erk1/2 and the BMP-Smad1/5/8 pathway. Tumor research in various tissues has shown that deletion of ID-1

leads to impaired angiogenesis and halting of tumor growth (187-190). Similarly, Klf4, a transcription factor with zinc-finger domain, is known to be expressed in endothelial cells, inducing proliferation and tube formation in response to VEGF, as well as protecting vascular integrity and regeneration during inflammation in response to BMPs (191-193).

1.4.3. Angiogenic signaling and nutrient sensing: the mechanistic Target of Rapamycin *(mTOR)- and AMP-activated serine/threonine protein kinase (AMPK)-pathway.*

All processes in the developing fetus are dependent on adequate energy supply. In the absence of nutrients, growth cannot take place. Therefore, nutrient sensing is extremely important for developmental processes such as angiogenesis. The mechanistic Target of Rapamycin (mTOR)-pathway controls cell growth in response to its environment (e.g. stress, oxygen, nutrient status) through protein synthesis as well as lipid, nucleotide, and glucose metabolism (194, 195). Eukaryotic translation initiation factor 4E-Binding Protein 1 (4E-BP1), a member of the family eukaryotic initiating factor 4E (eIF4E) binding proteins, is one of the main effectors activated by mTOR. eIF4E proteins bind to the 5´-cap recognition site on mRNA, preventing the recruitment of translation initiation complexes (196, 197). Phosphorylation of 4E-BP1 allows its binding to eIF4E, thereby preventing inhibition of larger translation initiation complexes for protein synthesis, and so promoting protein synthesis (197-199). Similarly, AMP-activated serine/threonine protein kinase (AMPK), is a sensor for nutrient status (mainly glucose), activated under starving conditions and suppressed when there is enough energy in the form of ATP in the cells (200). It has a predominant role in the inhibition of fatty acid and sterol synthesis, but also binds to Raptor, thereby inhibiting mTOR activity (figure 8)(195, 201). The mTOR pathway has recently been implicated in angiogenesis, primarily as a therapeutic target in cancer research (202). Multiple crosstalks between the mTOR-pathway and the VEGF-signaling pathway have been identified. For example, activation of the mTOR-pathway can increase VEGF via induction of HIF1a expression (203). Likewise, interaction between the mTOR- and BMP-signaling pathways have been shown in some cases as well. For example, insulin-like growth factor 1 (IGF-1) has been shown to suppress BMP-signaling in prostate cancer by activating mTOR-signaling and thereby dysregulating pro-proliferative and anti-apoptotic functions of the BMP-signaling (204). In the interest of this research project, mTOR and AMPK might provide a link between nutrient sensing and aberrant angiogenesis and lung development.

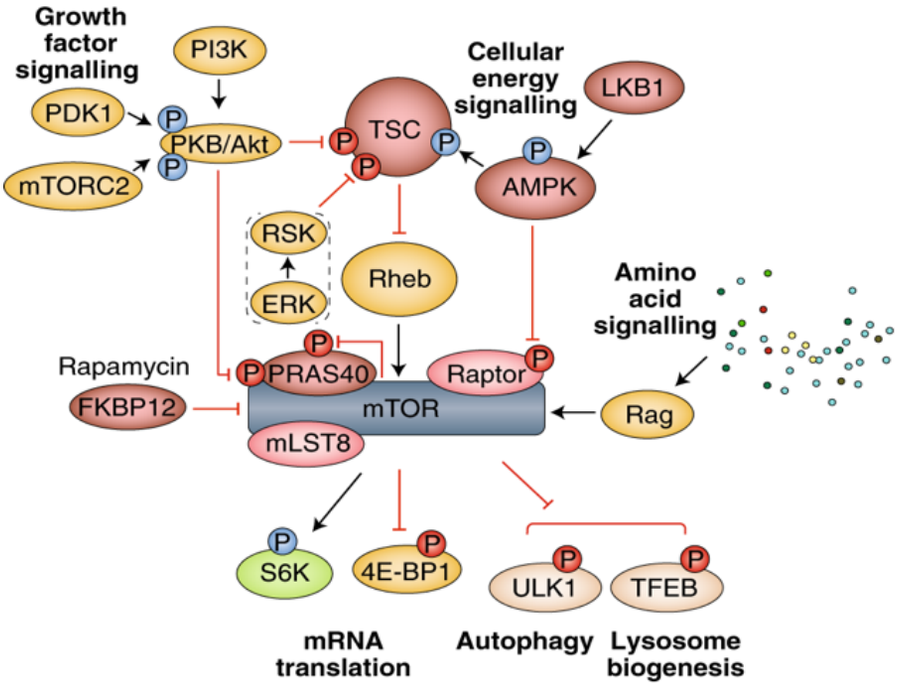

Figure 8: Major components and regulators of mTORC1 signaling. Key players in mTORC1 signaling, including upstream regulators that integrate growth factor and cellular energy signaling into mTORC1, and downstream major effectors that mediate mTORC1 effects on mRNA translation, autophagy and lysosome biogenesis are shown. Kim and Guan, 2019 (194). Reprinted with permission of Springer Nature, license number 4994720063646.

1.4.4. The influence of the extracellular matrix on pulmonary angiogenesis

The alveoli and the vessels are embedded in the ECM of the lung (literally, the space outside of the cells). This matrix provides the external structure and stability of 'soft' tissue like the vessels and conductive airways, as well as elasticity for the alveoli (205, 206). It is essential for cell function, growth and differentiation; changes in the ECM are associated with various lung diseases such as COPD, asthma, PAH and BPD (205, 207-210). The pulmonary ECM is composed of over 150 components that can be divided into two groups: the basement membrane that lines the endothelium and epithelium as a scaffold, and the interstitial matrix that provides the structural frame for the lungs (205, 211, 212). The main components are elastic fibers, collagen and connective proteins (211, 212).

The composition of the ECM determines its rigidity/elasticity, influencing the shape and function of the cells imbedded in it and therefore determining a large part of organ function (205, 206). The elastic fibers especially are a crucial factor regulating angiogenesis as well as alveolarisation. Elastin directs the outgrowth of primary and secondary septae, accumulating at the tips of these septae (23, 213). Studies have

shown a lack of alveolarisation and angiogenesis in mouse models with elastin haploinsufficiency, as well as platelet derived growth factor-A-deficiency (PDGF-A), which causes a reduction of elastic fibers organization (23, 213). The disruption of elastic fiber assembly at the tips of secondary septae has also been observed in neonatal chronic lung diseases (24, 25).

The communication between the vessels and ECM is not unidirectional: not only does the ECM influence vessel growth, the vessels also influence the composition of the ECM. For example, VEGF has been shown to induce metalloproteinase (MMP) expression in immortalized chondrocytes, an *in vitro* model for osteoarthritic inflammation (214). Likewise, an *in vitro* model for melanoma has shown that BMPs induce MMP expression as well (215). The MMPs are endoproteases that play a lead role in tissue and vascular remodeling (205, 216). MMP-2 and MMP-9 are gelatinases, found predominantly in vasculature, and can degrade collagen, elastin, fibronectin and various other ECM components. Interestingly, both MMP-2 and MMP-9 have binding capacity for TGFβ, member of the BMP family (216). In addition, it has been shown that MMP-$9^{-/-}$ mice exhibit a decreased level of VEGF, associated with decreased angiogenesis and tumor growth (217). Patients with COPD have elevated concentrations of MMP-2 that are positively correlated with severity of the emphysema (218). Similarly, an increase of MMP-2 was found in arterial smooth muscle cells in PAH (219). In asthma, however, where tissue remodeling is not a main aspect of disease, no effect on MMP-2 expression levels has been reported (218).

As has been demonstrated by previous studies, pulmonary angiogenesis is vital for alveolarisation, and is controlled by the expression of angiogenic growth factors as well as composition of the ECM. The process of angiogenesis can be adversely influenced by prenatal conditions as well as postnatal lung injury caused by mechanical ventilation and/or oxygen therapy, as has been studied extensively in models for BPD. This type of neonatal lung injury decreases the number of microvessels and capillaries in the lung (48, 220). As a result of dysregulated vessel formation, alveolarisation is reduced, thereby increasing the risk for acute and chronic lung diseases.

1.5. Studying IUGR

Ideally, the influence of IUGR on pulmonary angiogenesis is explored in humans. Since samples of newborn lungs are scarce and a controlled study is impossible, we used a rat model of IUGR in order to study angiogenic pathways.

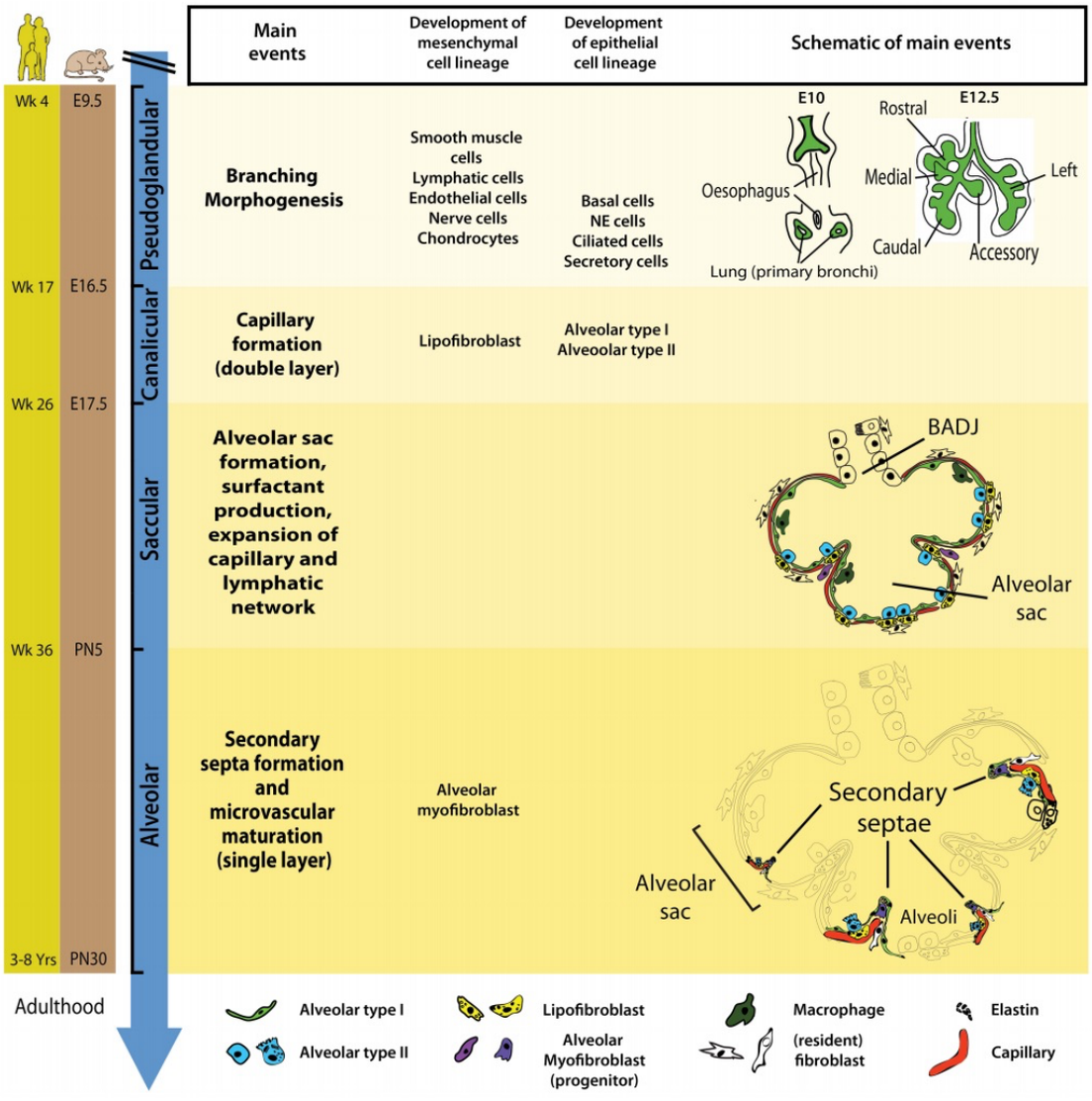

Figure 9: stages of pulmonary development in humans, as compared to rodents (PN=postnatal, E=embryonic)(221) © 2015 Chao, El Agha, Tiozzo, Minoo and Bellusci, distributed under the terms of the Creative Commons Attribution License (CC BY).

1.5.1. The animal model: a rat model of IUGR

A variety of animals are to be taken into consideration for studies that investigate and translate into human pathomechanisms. On the one side of the spectrum are the larger models, such as sheep and pigs. The advantage is the likeness to human intra- and extrauterine development and the size of the organs. The downside of these larger animals are the high costs related to the keeping of these animals and the complexity of procedures. On the other side of the spectrum, are the rodents (mainly mice, rats and rabbits) that come in to consideration. The clear advantage here are the lower costs for keeping these animals, the quick reproduction cycle and the option to hold and investigate larger groups simultaneously (i.e. under identical circumstances)(222). The disadvantage is that the pulmonary development does not completely align with the

development in humans (figure 9): rats are typically born with lungs in the middle-late saccular phase and mice in the early saccular phase. Their lungs are therefore comparable with those of premature human infants at birth (221). To minimize the disadvantages as well as profiting from the many advantages, we have chosen a rat model.

IUGR can be induced in rats in various ways, each corresponding or mimicking different causes for IUGR in humans. Several models have been established to study the impact of placental dysfunction on fetal development, including bilateral uterine ligation, uteroplacental embolization, the surgical removal of endometrial caruncles, single umbilical artery litigation, and hypoxic chambers to mimic placental dysfunction (223). In the present study, IUGR was induced using a maternal low protein diet (LP, Altromin C1003) during gestation, whereas the control dams are fed normal protein (NP, Altromin C1000) diet. After birth, dams of both groups receive standard chow. The rat pups of LP-fed dams are born with low birth weight and exhibit a postnatal catch-up growth. This rat model of IUGR is well documented and established in our research group(108, 131-133, 139, 224-227).

1.6. Hypothesis and goals

Neonatal chronic lung disease (CLD, BPD) is characterized by alveolar and microvascular hypoplasia. Angiogenesis has been recognized to play a crucial role in alveolarisation and is reduced in lungs with CLD (BPD). Moreover, IUGR results in a loss of alveolarisation and an increased risk of CLD, independent of prematurity. Initial studies show that IUGR adversely affects postnatal lung endothelial cell function and micro-vascular formation. However, to date it has not been investigated whether IUGR itself causes an intrauterine and postnatal dysregulation of angiogenesis. Therefore, I hypothesized that IUGR disrupts pro-angiogenic and nutrient sensing signaling, and matrix remodeling during intrauterine and postnatal lung development, thereby adversely affecting angiogenesis.

To test this hypothesis, I defined three specific aims:

(1) To investigate micro-vascular formation in the lungs by vessel quantification and assessment of mRNA and protein expression of vessel markers.

(2) To study pro-angiogenic (including VEGF- and BMP- pathway) and nutrient sensing signaling (AMPKα and mTOR pathway) using qRT-PCR and western blotting for the measurement of mRNA and protein expression, respectively.

(3) To study elastic fiber assembly and proteolytic activity in lungs after IUGR using histological staining and zymography.

The hypothesis will be tested in a rat model for IUGR, induced by a low protein diet, and compared with control animals that have received a normal diet during gestation. My own work in this animal model will be complemented with human data from Mendelian Randomization studies, to provide a translational link from the rat to the human.

2. CUMULATIVE PART OF THE DISSERTATION

The contents of this chapter were published:

Kuiper-Makris C, Zanetti D, Vohlen C, Fahle L, Muller M, Odenthal M, Felderhoff-Müser U, Dötsch J, Alejandre Alcazar MA. Mendelian randomization and experimental IUGR reveal the adverse effect of low birth weight on lung structure and function. Sci Rep 10, 22395 (2020). https://doi.org/10.1038/s41598-020-79245-7.

scientific reports

Check for updates

OPEN

Mendelian randomization and experimental IUGR reveal the adverse effect of low birth weight on lung structure and function

Celien Kuiper-Makris[1,2], Daniela Zanetti[3,4], Christina Vohlen[1,2], Luise Fahle[1,2], Marion Müller[5], Margarete Odenthal[5,6], Ursula Felderhoff-Müser[7], Jörg Dötsch[2] & Miguel A. Alejandre Alcazar[1,6,8,9]✉

Intrauterine growth restriction (IUGR) and low birth weigth (LBW) are risk factors for neonatal chronic lung disease. However, maternal and fetal genetic factors and the molecular mechanisms remain unclear. We investigated the relationship between LBW and lung function with Mendelian randomisation analyses and studied angiogenesis in a low protein diet rat model of IUGR. Our data indicate a possible association between LBW and reduced FEV1 (p = 5.69E−18, MR-PRESSO) and FVC (6.02E-22, MR-PRESSO). Complimentary, we demonstrated two-phased perinatal programming after IUGR. The intrauterine phase (embryonic day 21) is earmarked by a reduction of endothelial cell markers (e.g. CD31) as well as mRNA expression of angiogenic factors (e.g., Vegfa, Flt1, Klf4). Protein analysis identified an activation of anti-angiogenic mTOR effectors. In the postnatal phase, lung capillaries (< 20 µm) were significantly reduced, expression of CD31 and VE-Cadherin were unaffected, whereas SMAD1/5/8 signaling and Klf4 protein were increased (p < 0.01). Moreover, elevated proteolytic activity of MMP2 and MMP9 was linked to a 50% reduction of lung elastic fibres. In conclusion, we show a possible link of LBW in humans and reduced lung function in adulthood. Experimental IUGR identifies an *intrauterine phase* with inhibition of angiogenic signaling, and a *postnatal phase* with proteolytic activity and reduced elastic fibres.

Intrauterine growth restriction (IUGR) is a multifactorial disease affecting approximately 10% of the newborn population and is classically defined as a birthweight two standard deviations below the 50th percentile for the gestational age[1]. Recently, IUGR has been characterised as a failure of a foetus to reach its full growth potential due to genetic or environmental factors, including maternal, placental, and fetal causes, which then leads to restricted nutrient and oxygen supply[1,2]. Infants with IUGR are at higher risk for adverse perinatal complications, such as prematurity, respiratory distress, and bronchopulmonary dysplasia (BPD)[2]. It has also been demonstrated that IUGR followed by postnatal catch-up growth increases the susceptibility to chronic adult's diseases beyond infancy, including hypertension and vasculopathies[3].

[1]Translational Experimental Pediatrics-Experimental Pulmonology, Department of Pediatric and Adolescent Medicine, Faculty of Medicine and University Hospital Cologne, University of Cologne, Kerpener Strasse 62, 50937 Cologne, Germany. [2]Department of Pediatric and Adolescent Medicine, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany. [3]Division of Cardiovascular Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA. [4]Stanford Cardiovascular Institute, Stanford University, Stanford, CA, USA. [5]Institute of Pathology, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany. [6]Center for Molecular Medicine Cologne (CMMC), Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany. [7]Department of Paediatrics I, University Hospital Essen, University Duisburg-Essen, Essen, Germany. [8]Institute for Lung Health, Member of the German Centre for Lung Research (DZL), University of Giessen and Marburg Lung Centre (UGMLC), Giessen, Germany. [9]Cologne Excellence Cluster for Stress Responses in Ageing-Associated Diseases (CECAD), Cologne, Germany. ✉email: miguel.alejandre-alcazar@uk-koeln.de

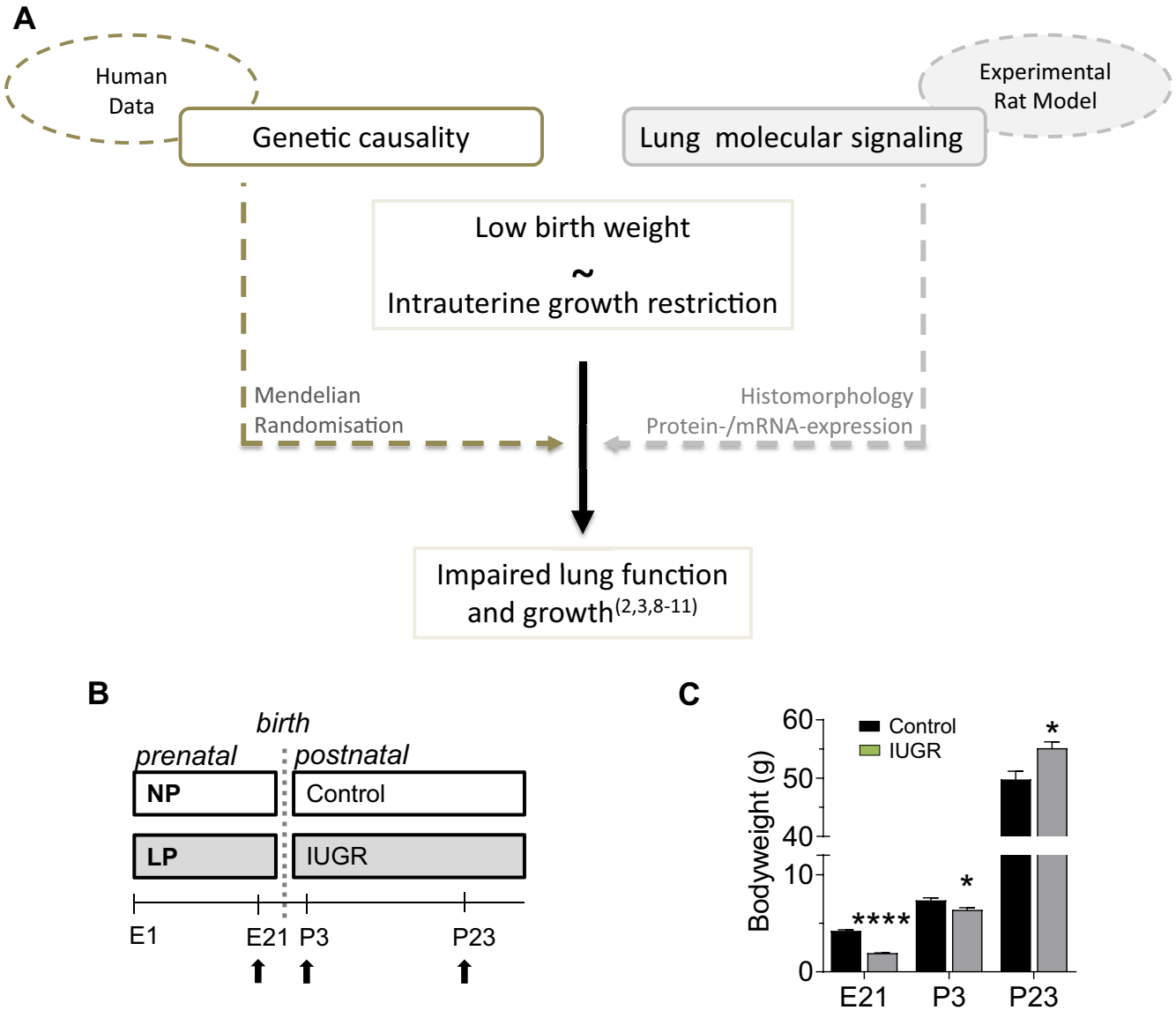

Figure 1. Schematic representation of the study layout, using Mendelian randomisation (left) as a strategy to study the genetic origin of low birth weight, vs. using molecular methods to study the effect of intrauterine growth restriction (IUGR) on lung development (right) (**A**). Timeline of the experimental model in which IUGR is induced with a low protein diet (LP) during gestation in Wistar rats; control dams received normal protein diet (NP). Lungs were harvested at embryonic day 21 (E21), postnatal day 3 (P3) and P23 (**B**). Measurements provide a quantification of the mean body weight of the control and IUGR group on time points E21, P3, and P23 (**C**). Mean ± SEM (n = 10/group). A non-parametric T-test was used to compare IUGR to the control group, *$p < 0.05$, ****$p < 0.0001$.

Intrauterine exposure to adverse nutritional, metabolic, and hormonal alterations can interfere with organ development and induce life-long changes in structure and physiology. This was initially described as fetal, perinatal or metabolic programming by Barker and colleagues in the 1990s[4,5]. Previous studies have shown that IUGR impairs alveolar formation and lung growth, leading to reduced lung function in adult rats. These structural and functional changes may be related to a disruption of key developmental signaling pathways, such as TGFβ signaling, and inflammatory response. This may induce matrix remodeling, including distorted elastic fibre assembly[6,7]. While clinical studies associated IUGR with lower diffusion capacity, increased susceptibility to infections, and obstructive pulmonary disease[8–10], the genetic disposition remain elusive. Recent studies demonstrated that low birth weight (LBW), used as a proxy for IUGR, is causally related to an increased susceptibility to coronary artery disease, and hightens the risk for pulmonary arterial hypertension (PAH) in infants with BPD[11]. These clinical data suggest a causual genetic association of IUGR and susceptibility to lung diseases that needs to be elucidated.

Angiogenesis is essential for alveolar formation and lung growth. The activation of endogenous pro-angiogenic pathways increases endothelial cell survival, proliferation and migration, thereby driving alveolarisation[12]. The concerted interaction of growth factor signaling, such as vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP) signaling, is central in endothelial cell function[13,14]. The disruption of these critical angiogenic pathways impairs microvessel formation and arrests lung growth, as is seen in BPD[13,15].

Previous studies have shown that IUGR decreases pulmonary vessel growth in sheep and impairs proliferation, migration, and tube formation of pulmonary artery endothelial cells[16]. However, the molecular mechanisms disrupting developmental processes and angiogenesis in lungs following IUGR remain unclear.

Based on the association of IUGR resulting in LBW and BPD we pursued two approaches: Firstly, we studied the long-lasting causal relationship of LBW and reduced lung function in humans with Mendelian randomisation; secondly, we investigated whether disruption of developmental pathways at intrauterine and postnatal time points contributes to impaired angiogenesis and elastic fibre metabolism after IUGR, thereby causing failed alveolarisation and functional impairment (Fig. 1A).

Materials and methods

Mendelian randomisation. We performed two-sample Mendelian randomisation analyses to infer causality between birth weight and forced expiratory volume in 1 s (FEV1) and forced vital capacity (FVC), used as proxies for lung function. We selected and used as exposure the genome-wide significant independent hits (p value ≤ 5E10−8) associated with own and offspring birth weight (Supplemental Table 1) from the genome-wide association meta-analyses of own and offspring birth weight performed by the Early Growth Genetics (EGG) Consortium[17]. We used the genome-wide association studies (GWAS) summary statistics of FEV1 (field ID 3063) and FVC (field ID 3062) performed in the UK Biobank by Neale et al. (http://www.nealelab.is/uk-biobank/) as outcomes.

We used four separate methods to estimate causal effects: the standard inverse-variance weighted (IVW) regression with and without MR-PRESSO[18] (Mendelian Randomization Pleiotropy RESidual Sum and Outlier; to minimise the risk of horizontal pleiotropy); as well as two robust regression methods, the weighted median-based method, and Egger regression[19]. We applied robust methods with special assumptions about the behavior of pleiotropic variants, such as MR-Egger[20], which assumes pleiotropic effects are uncorrelated with the genetic associations with the risk factor, the InSIDE assumption; and the MR-PRESSO[18], that excludes outlying variants as being potentially pleiotropic. In addition, we performed leave-one-out sensitivity analyses to identify if a single SNP was driving an association. We estimated statistical power for the MR analyses assuming a clinically relevant fixed effect size of 0.1 SD with an alpha threshold of 0.05. Power for MR analyses was estimated using the method reported by Burgess et al.[21,22]. We performed the two-sample MR analyses[19,23] with the R package *TwoSampleMR*.

Animal studies. All animal procedures were performed as previously described[6,7], in accordance with the German regulations and legal requirements and approved by the local government authorities (Regierung von Mittelfranken, AZ 621-2531.31-11/02 and AZ 621-2531.31-14/05)[24]. Three time points were investigated: (1) Embryonic day 21 (E21): caesarean section was performed and the foetuses were euthanised; (2) Postnatal day 3 (P3); (3) P23; 2–6 dams for each group. n = 10 for each experimental group. Lungs were excised *enblock* and either snap frozen and stored − 80 °C or fixed with 4% paraformaldehyde for paraffin embedding as previously described[7].

Tissue assays. *RNA extraction and real-time qPCR.* Total RNA extraction and quantitative RT-PCR were performed as previously described[7]. Quantitative changes in mRNA expression were assessed with Taqman or SYBR Green PCR Master Mix (Invitrogen, 11743500 & 11760500, Germany) using a 7500 Real-Time PCR System (Applied Bioscience). Primers were designed using Primer Express Software (Applied Biosystems) (Supplemental Table 2). The $\Delta\Delta C_t$ method, as previously described, was used for quantification[25].

Protein extraction, quantification, and immunoblot. Protein was isolated from homogenised whole-lung tissue and quantified, followed by immunoblotting as previously described[7]. The primary antibodies and the secondary peroxidase-conjugated anti-mouse, anti-rabbit or anti-rat antibodies (commercially available and tested) are listed in Supplemental Table 3. Quantitative analysis was performed with densitometry (Bio-Rad ImageLab software, Bio-Rad, Munich, Germany) using hypoxanthine–guanine phosphoribosyltransferase (HPRT) and β-Actin as loading controls. All data represent contiguous lanes, complete blots are shown in the Supplemental Figures.

Zymography. Protease activity of metalloproteinase 2 (MMP2) and MMP9 was analysed by gelatin-based zymography as previously described[7]. Activity was quantified by densitometric analysis on negative images (Image Lab Software, Bio-Rad Laboratories, Germany). All data represent contiguous lanes, blots are not cropped.

Histology and immunhistochemistry. *Microvessel count.* Randomly selected lung sections (3 µm thickness) were stained with the primary antibody anti-von Willebrand Factor, followed by a secondary antibody (for details see Supplemental Table 3). The sections were then scanned using the slide scanner (Leica SCN400). Microvessels (2–20 µm and 20–100 µm, displaying a lumen) were quantified in a total of ten fields of 20× view per tissue section and 6 random tissue sections per animal (6 animals per group).

Elastic fibre quantification. Lung sections were stained for elastic fibres using Resorcin Fuchsin from Weigert (Weigert's Iron Resorcin and Fuchsin Solution; Carl Roth, X877.3)[26], and counterstained yellow with Tatrazine [0.5% in 0.25% acetic acid (Dianova, cat. no. TZQ999, USA)]. The elastic fibre density as an index of parenchymal elastin content was analysed in up to ten fields of 20× view per tissue section and 6 random tissue sections

	FVC				FEV1			
Method	nsnp	b	se	p	nsnp	b	se	p
Own birthweight								
IVW	220	0.214	0.024	**7.79E−20**	220	0.185	0.022	**4.56E−17**
MR-PRESSO	174	0.151	0.014	**6.02E−22**	180	0.132	0.014	**5.69E−18**
MR Egger	220	0.264	0.064	**5.59E−05**	220	0.218	0.060	**3.47E−04**
Weighted median	220	0.091	0.014	**1.07E−10**	220	0.059	0.014	**3.86E−05**
Offspring birthweight								
IVW	118	0.193	0.032	**2.25E−09**	118	0.183	0.030	**1.50E−09**
MR-PRESSO	82	0.121	0.017	**3.67E−10**	87	0.110	0.017	**2.95E−09**
MR Egger	118	0.276	0.102	**7.57E−03**	118	0.284	0.095	**3.42E−03**
Weighted median	118	0.067	0.017	**6.13E−05**	118	0.067	0.018	**2.40E−04**

Table 1. Mendelian randomisation analyses of birth weight with expiratory volume in 1 s (FEV1) and forced vital capacity (FVC). The estimates represent SD change in outcome variable per SD change in the exposure tested. P-values significant after Bonferroni correction ($p \leq 1.25*10^{-1}$) are bold. se, standard error; P, p-value; nsnp, number of single-nucleotide polymorphism; IVW, inverse variance weighted method.

per animal (6 animals per group). Measurement was performed using Cell D 3.4 Olympus Soft Imaging Solutions (Olympus; CellSens, Germany).

Statistical analysis. All results are displayed as mean ± SEM. The unpaired non-parametric T-test was used to compare the control (normal protein) to the IUGR (low protein) group. For a quantitative comparison between time points, a two-way ANOVA followed by Bonferroni post-test was performed. The p-value was adjusted using Bonferroni post-test and less than 0.05 was considered significant. The statistical analyses were carried out using the Graph Pad Prism software (GraphPad software Version 6.0 and 7.0, San Diego, CA, USA).

Results

MR analyses indicate a possible association of birth weight with adult lung function. With more than 80% statistical power our data suggest an association of birth weight with lung function (FVC and FEV1) in all Mendelian randomisation analyses performed. The leave-one out sensitivity analysis did not highlight any SNPs with a large effect on the results. After excluding outlying variants as being potentially pleiotropic using MR-PRESSO, the analysis showed neither significant heterogeneity or directional horizontal pleiotropy. The analyses using MR-Egger and weighted median methods consistently yielded similar effect estimates. The direction of the effect was positive (i.e., LBW was associated with reduced lung function). Full results are shown in Table 1 and Supplemental Figure 1.

IUGR was established in a low-protein diet rat model. Low protein diet of dams during gestation induced IUGR in the offspring when compared to controls. The experimental rat model of IUGR is illustrated in Fig. 1B. As shown in Fig. 1C, offspring had LBW at (E21) and P3, whereas their weight was significantly higher at P23, indicating a postnatal catch-up growth after IUGR.

IUGR alters pulmonary vessel growth and regulates endothelial cell markers. Quantification of lung microvessels with CD31 immunostaining at P3 and P23 (Fig. 2A) revealed a significant formation of larger microvessels (20–100 µm) between P3 und P23 in control pups, not visible in IUGR. Small microvessels (< 20 µm) were significantly decreased in IUGR when compared to control at P23, suggesting dysfunctional postnatal angiogenesis after IUGR (Fig. 2B). The mRNA expression of *Pecam1* (CD31) was significantly reduced at E21 and immunoblot showed a non-significant reduction of VE-Cadherin protein expression at P3, but not at P23 after IUGR, compared to control (Fig. 2C,D).

IUGR dynamically regulates the pro-angiogenic VEGF pathway in lungs. The mRNA expression of the ligand *Vegfa* as well as its receptors, VEGF-R1 (*Flt1*) and VEGFR2 (*Flk1*), were significantly reduced in lungs after IUGR at E21, but not at P3 and P23 (Fig. 3A). The analysis of the VEGF-signaling pathway ERK1/2 revealed a significant reduction of total ERK1/2 at E21, with slightly increased phosphorylated ERK1/2 (pERK1/2) relative to total ERK1/2. Early postnatal (P3), IUGR increased total ERK1/2 protein as well as pERK1/2. Finally, we detected an inhibition of pERK1/2 in lungs after IUGR when compared to controls at the late postnatal stage (P23) (Fig. 3B).

IUGR dysregulates the pro-angiogenic BMP signaling pathway in lungs. BMP-receptor1a (*Bmpr1a*) was significantly reduced at E21, whereas *Bmpr1b* mRNA expression was significantly reduced after IUGR at P23. *Bmpr2* mRNA was also expressed differentially, with a slight reduction at E21 and a significant upregulation at P3 after IUGR (Fig. 4A). Immunoblot of the downstream signaling cascade of BMPRs showed activation of SMAD1/5/8 (pSMAD1/5/8) after IUGR at P3, but no difference at P23 (Fig. 4B). The mRNA expres-

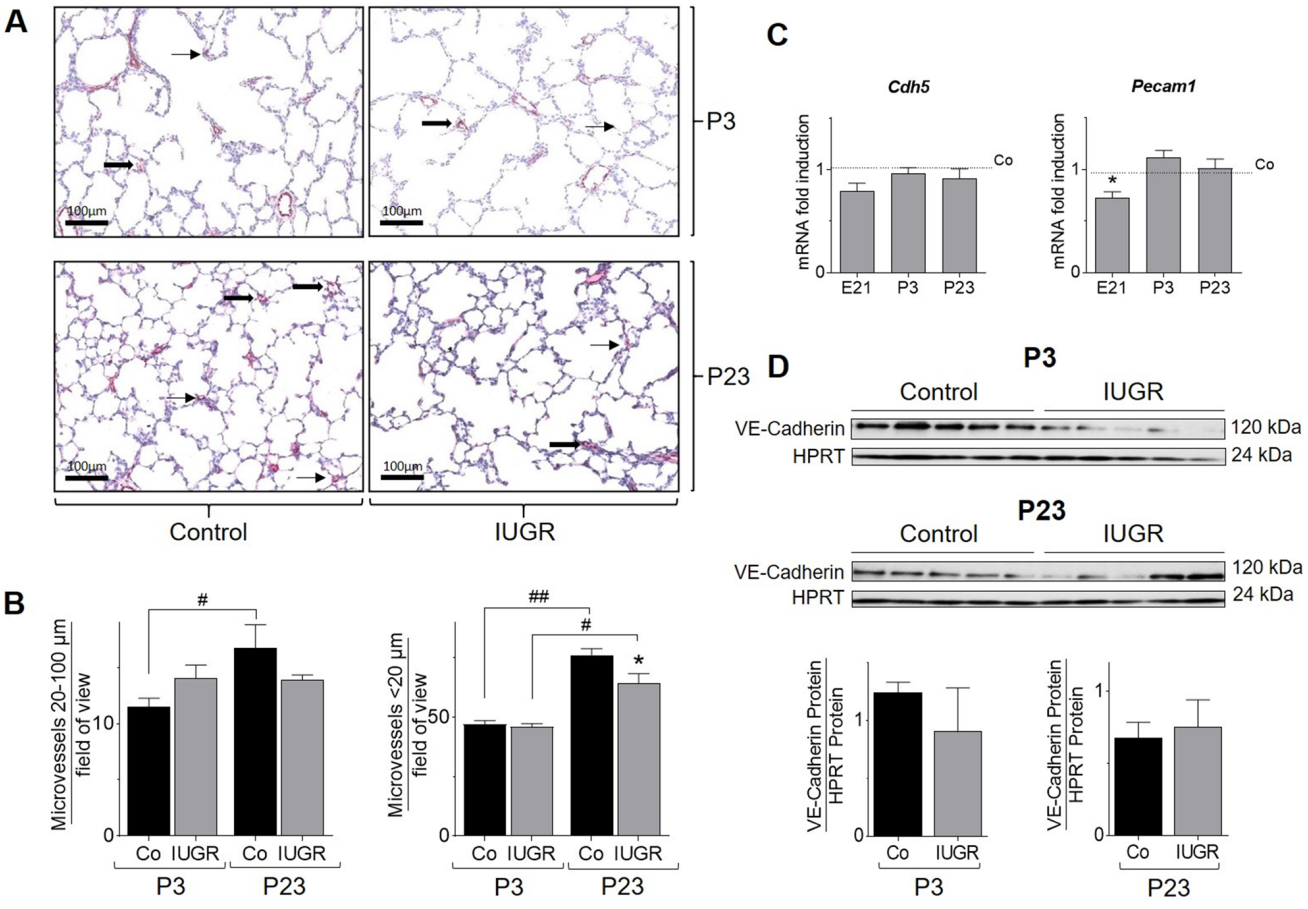

Figure 2. Intrauterine growth restriction (IUGR) impairs angiogenesis. (**A**, **B**) Representative images (×20 magnification) of pulmonary microvessels (0–100 µm) on postnatal day 3 (P3), and on postnatal day 23 (P23). Random lung sections were stained with von Willebrand Factor as an indicator of endothelial cells, followed by counting of microvessel count for vessels with a diameter of 20-100 µm (marked with ➡), and vessels with a diameter < 20 µm (marked with ⟶). The respective quantification is shown below the immunhistochemical stainings (n = 6/group). (**C**) Assessment of mRNA expression of pulmonary endothelial cell markers, VE-Cadherin (*Cdh5*) and CD31 (*Pecam1*) on embryonic day 21 (E21), P3 and P23 using qRT-PCR; Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) served as housekeeping gene; the control group was set at 1. (**D**) Measurement of protein abundance using immunoblot; the respective densitometric quantification of protein expression of VE-Cadherin on P3 and P23 is shown below the immunblots; VE-Cadherin was related to the loading control HPRT (n = 5–6/group). Mean ± SEM. A non-parametric T-test was used to compare IUGR to the control group,*$p < 0.05$. Comparison between P3 and P23 with a two-way ANOVA test, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

sion of apelin (*Apln*), inhibitor of differentiation (*Id1*), and Krüppel-like factor 4 (*Klf4*), regulators of endothelial cell homeostasis or BMP interactors, were also reduced after IUGR at E21. *Apln* remained significantly lower in IUGR than Control at P3, whereas *Klf4* gene expression was significantly downregulated at P23 (Fig. 4C). The protein abundance of Klf4, a regulator of stem cell capacity, was slightly reduced at E21 and more than twofold increased at P23 (Fig. 4D).

IUGR dysregulates cell metabolism through AMPKα and mTOR signaling. Immunoblot analysis revealed that IUGR significantly inhibited AMPKα signaling during the intrauterine phase (E21) (Fig. 5A). In contrast, assessment of mTOR signaling using 4E-BP1 as a downstream effector showed a marked activation at E21 (Fig. 5B). However, during postnatal lung development neither AMPKα nor mTOR signaling were significantly regulated by IUGR.

IUGR increases lung protease activity and reduces lung elastic fibre content. A Hart stain for elastic fibres (Fig. 6A) revealed a reduction of relative elastic fibre content by 50% at both time points P3 and P23 (Fig. 6B). Assessment of *Eln* (elastin) mRNA expression did not significantly differ between IUGR and Control group (Fig. 6C), suggesting increased degradation as a result of proteolytic activity. To test this notion we used zymography, and determined a significant increase of metalloproteinase 2 (MMP2) and MMP9 activity after IUGR at P3 (Fig. 6D).

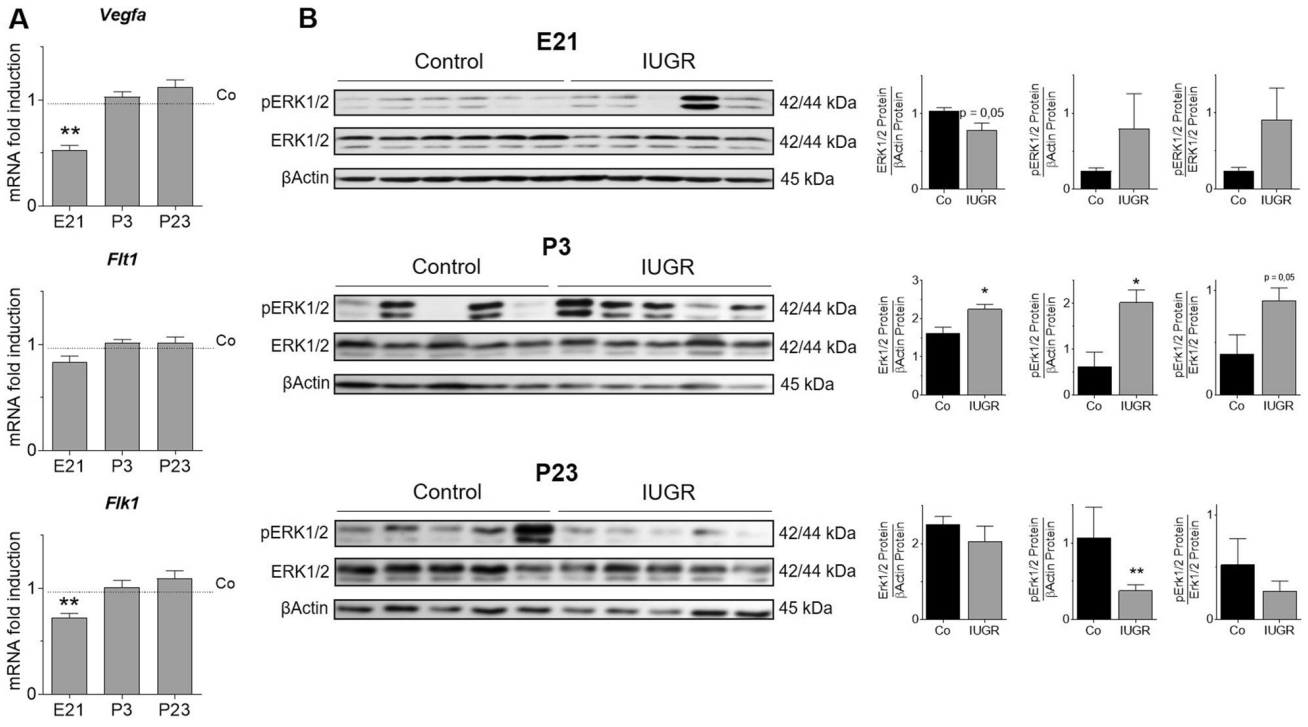

Figure 3. IUGR dysregulates gene expression of components and the activation of vascular endothelial growth factor (VEGF) signaling in lungs. (**A**) Assessement of *Vegfa*, VEGF-Receptor 1 (*Flt1*) and VEGF-R2 (*Flk1*) on embryonic day (E21), postnatal day 3 (P3), and P23 using qRT-PCR; Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) served as housekeeping gene; the Control group was set at 1 (n = 10/group). (**B**) Immunoblots illustrating protein abundance of total ERK1/2 and phosphorylated ERK1/2 (pERK1/2) on E21, P3 and P21; pERK1/2 was related to βActin or to total ERK1/2; the densitometric analysis is shown next to the respective immunoblot (n = 5–6/group). Mean ± SEM. A non-parametric T-test was used to compare IUGR to the control group,*$p < 0.05$, **$p < 0.01$.

Discussion

The present study shows a possible association between LBW in humans, used as a proxy of IUGR, and reduced lung function in adulthood. In addition, it provides a pathomechanism of acute and long-term impact of IUGR on pulmonary microvascular formation and elastic fibre formation in an experimental rat model of IUGR. The combined results of these two approaches paint the picture of dynamic intrauterine programming of pulmonary development. Specifically, during the *intrauterine phase,* restricted nutrient supply and cellular stress are related to a significant disruption of pro-angiogenic VEGFA- and BMP-signaling, activation of anti-angiogenic mTOR signaling, downregulation of angiogenic growth and transcription factors, and loss of endothelial cell markers. Whereas during the *postnatal phase*, angiogenic signaling caught up, but small microvessels were significantly reduced and associated with an increased lung proteolytic activity, resulting in a persistent reduction of elastic fibre content. These processes accumulate in a loss of microvasculature and alveolarisation, evidenced by a loss of lung function in adulthood.

Linking LBW to reduced lung function using Mendelian randomization. With Mendelian randomisation, we have shown a positive association between LBW and reduced lung function (FEV1, FVC), indicating a possible reduced functional lung volume in individuals with LBW. It has been previously shown that a reduction of FVC is associated with lung emphysema[27], defined as a decrease in elastic recoil[28]. In addition, previous studies have shown an association between LBW and type 2 diabetes and cardiovascular diseases[11]. Our data provide a direct link between maternal effects and LBW-associated lung function that is in line with the Barker Hypothesis.

Strengths and limitations of Mendelian randomization. Our study included the large sample size and the most recent and powerful GWAS summary statistics used as exposures and outcomes. In addition, we used a wide range of sensitivity analyses increasing robustness of our findings. Our study also has several limitations. First, the participants included in the genetic analyses were of European ancestry. Hence, our results may not be generalizable to other ethnic groups with significantly different prevalence/predispositions with regard to the outcome. Second, we analyzed birth weight as a continuous variable without specifically looking at LBW. Moreover, we cannot exclude the importance of additional environmental factors implicated in the relationship between birth weight and lung function. Finally, samples of the GWAS for the exposure (the genome-wide association meta-analyses of own and offspring birth weight) and the GWAS for the outcome [FEV1 (field ID

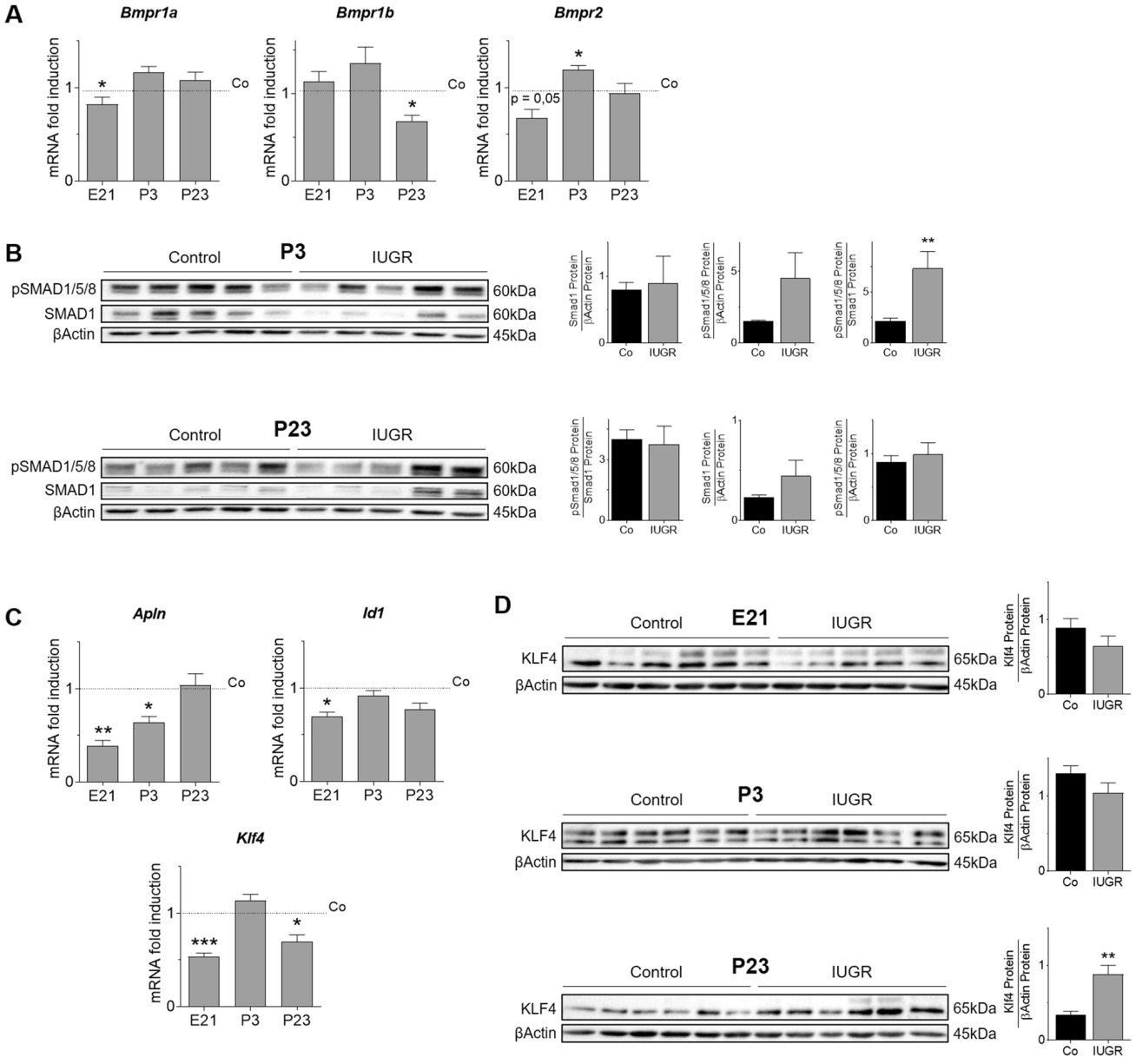

Figure 4. IUGR dysregulates gene expression and activation of the bone morphogenetic protein (BMP)-signaling pathway components. (**A**) Assessement of gene expression of BMP-Receptor 1a *(Bmpr1a), Bmpr1b* and *Bmpr2* on embryonic day (E21), postnatal day 3 (P3), and P23 using qRT-PCR; Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) served as housekeeping gene; the Control group was set at 1 (n = 10/group). (**B**) Immunoblots showing protein abundance of phosphorylated SMAD1/5/8 (pSMAD1/5/8) and total SMAD1 on P3 and P23; βActin served as loading control; pSMAD1/5/8 was related to βActin or to total SMAD1; densitometric summary data are shown next to the respective immunoblot (n = 5–6/group). (C) Measurement of mRNA expression of angiogenic transcription factors apelin (*Apln*), inhibitor of differentiation 1 (*Id1*), and Krüppel-like factor 4 (*Klf4*); *Gapdh* served as housekeeping gene; the Control group was set at 1 (n = 10/group) (**C**), as well as the protein expression of transcription factor Klf4 (**D**), with densitometric quantification shown below (n = 5–6/group). Mean ± SEM. A non-parametric T-test was used to compare IUGR to the control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3063) and FVC (field ID 3062) performed in the UK Biobank] have in part some overlaps and could thereby potentially bias our results.

Translational approach: dysregulation of angiogenic signaling is associated with impaired lung growth in rats with IUGR. VEGF promotes angiogenesis and alveolarisation in animal models of neonatal lung injury[29]. In the present study, we demonstrate a significant downregulation of *Vegfa* and *Flk1* (VEGF-R2) in the intrauterine phase, which is followed by a catch-up to reach Control levels. Interestingly, the downstream effector ERK1/2 protein[30] is reduced on E21, and increased after birth. However, the activation of

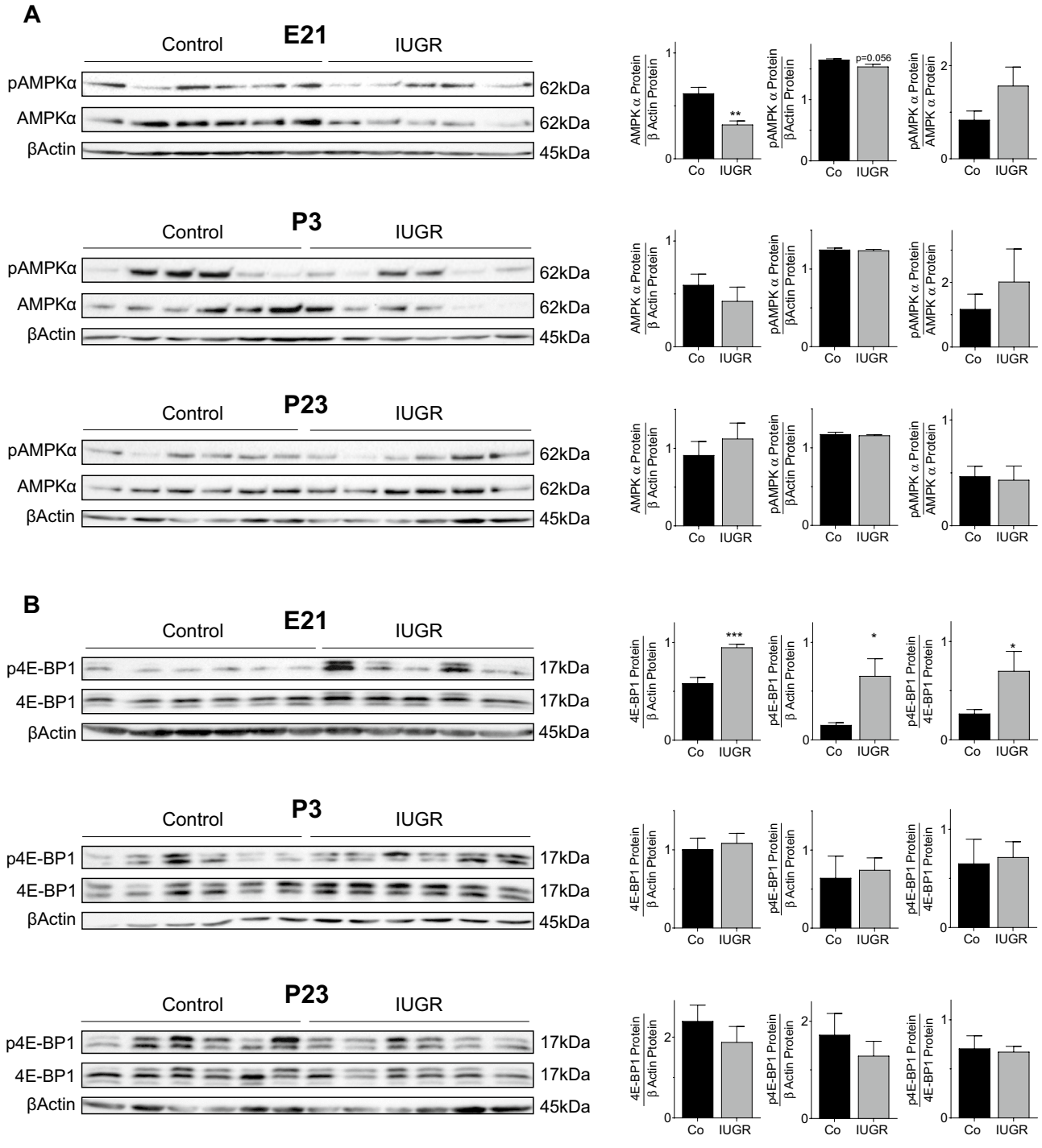

Figure 5. IUGR dysregulates nutrient-sensing signaling pathways that are involved in angiogenesis. (**A**) Immunoblots showing the analysis of AMP-Activated protein kinase (AMPK)α-pathway in total lung homogenate on embryonic day (E21), postnatal day 3 (P3), and P23; total AMPKα and phosphorylated AMPKα (pAMPKα) were assessed; βActin served as loading control; pAMPKα was related to β-Actin or to total AMPKα; densitometric summary data are shown next to the respective immunoblot (n = 6/group). (**B**) Total 4E-BP1 and phosphorylated 4E-BP1 (p4E-BP1) as a downstream effector of mTOR-pathway were assessed with immunoblot on E21, P3, and P23; βActin served as loading control; p4E-BP1 was related to βActin or to total 4E-BP1; densitometric summary data are shown next to the respective immunoblot (n = 6/group). Mean ± SEM. A non-parametric T-test was used to compare IUGR to the Control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ERK1/2 remains reduced at P23, suggesting a possible programming of intracellular VEGF-A resistance. Moreover, VEGF signaling is also activated by AMPK, a pathway that promotes endothelial cell differentiation, pro-

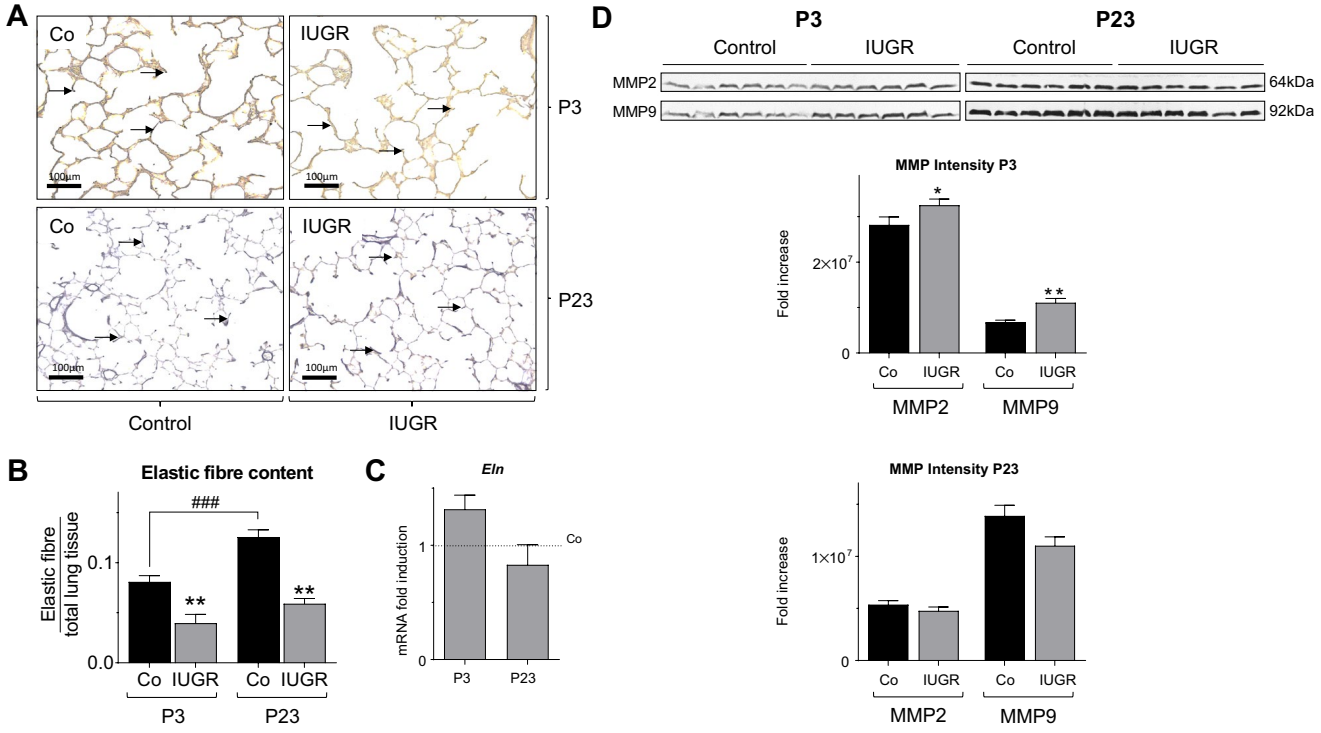

Figure 6. IUGR activates lung proteolytic activity and reduces lung elastic fibre content. (**A**) Representative images (20x magnification) of Hart stained lung at postnatal day 3 (P3) and P23, depicting elastic fibres as indicated with black arrows. (**B**) The quantification of elastic fibre content, relative to total lung tissue at P3 and P23 (n = 6/group). (**C**) Measurement of Elastin (*Eln*) mRNA expression on P3 and P23 using qRT-PCR analysis; Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) served as housekeeping gene; the Control group was set at 1 (n = 10/group). (**D**) Analysis of the proteolytic activity of matrix metalloprotease 2 and 9 (MMP2, MMP9) at P3 and P23 using zymography. Both MMP2 and MMP9 are regulators of matrix remodelling; the densitometric quantification of the zymography is shown below (n = 6/group). Mean ± SEM. A non-parametric T-test was used to compare IUGR to the control group, *p<0.05, **p < 0.01. Comparison between P3 and P23 with a two-way ANOVA test, ###p < 0.001.

liferation and migration[31]. Reduced activation of the AMPKα signaling pathway during the intrauterine period may further contribute to the inhibition of the VEGF signaling machinery.

BMP signaling drives angiogenesis and is essential for the maintenance of endothelial cell homeostasis, and mutations in this family are associated with hereditary PAH. The transduction of BMP signaling is facilitated by complex formation between BMP receptors 1 and 2, activating its downstream canonical SMAD1/5/8 and non-canonical ERK1/2 signaling[14]. Our data show that IUGR significantly downregulates *Bmpr1a* and *Bmpr2* during the intrauterine period, whereas *Bmpr1b* was unaffected. In the postnatal phase, we found opposing effects, with similar gene expression of *Bmpr1a* and *Bmpr2*, but significant lower *Bmpr1b* in IUGR than control lungs. IUGR may block the proliferative Bmpr1a/Bmpr2 pathways during the intrauterine phase, while it is shifted to pro-proliferative signaling by postnatal reduction of *Bmpr1b* as an attempt to promote vascularisation and compensate for the intrauterine growth restriction[32]. This notion is further sustained by a postnatal activation of the downstream effector pSMAD1/5/8. This early developmental blockade of VEGF and BMP-receptor signaling in the lung may impair angiogenesis, induce arrest of alveolarisation and contribute to early origin of chronic lung diseases, such as BPD and PAH.

To determine whether the disruption of developmental signaling after IUGR may be related to changes in growth and transcription factors, we assessed *Id1*, *Apln*, and *Klf4* expression. In accordance with our previous data, *Id1*, *Apln*, and *Klf4* were significantly reduced by 50% in fetal lungs with IUGR, and postnatally upregulated. Downregulation of these factors has been associated with the pathogenesis of PAH[15,33,34]. The growth factor apelin is pro-angiogenic and regulates endothelial cell migration, proliferation and survival[15], and preserves lung growth in a model of BPD[35]. Similarly, the expression of *Id1* is induced by BMPR2 signaling, and maintains endothelial cell function[33,36]. On the other hand Klf4, promotes angiogenesis through activation of VEGF signaling[34], and regulates cell survival and differentiation. Loss of these angiogenic factors may impair vascular development and induce chronic vascular lung disease.

Linking nutrient sensing pathways to angiogenic signalling in lungs of rats with IUGR. The pathways that signal anti-angiogenic effects during lung development remain uncertain. Organ growth requires an increased metabolic rate, which requires oxygen and nutrient supply. Nutritive restriction or placental insufficiency forces adaptation of the metabolism by changing cellular energy consumption. The serine/therosine kinase, mammalian target of rapamycin (mTOR) senses nutrient status in growing vessels, and modulates cel-

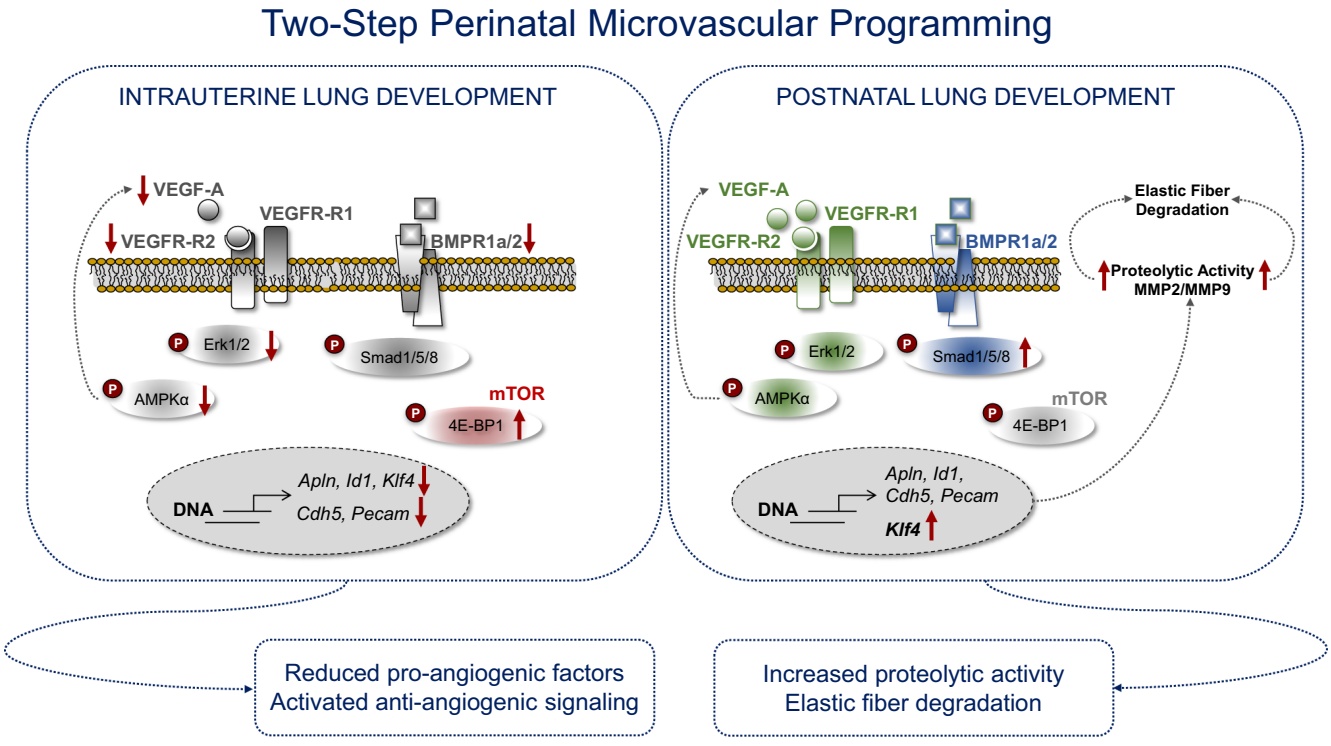

Figure 7. Speculative working model of two-step perinatal microvascular programming after intrauterine growth restriction (IUGR) with low birth weight (LBW). Fetal genetic factors contribute to low birth, linked to reduced lung function later in life. *Intrauterine phase*: IUGR inhibits angiogenic signaling, including vascular endothelial growth factor (VEGF), AMP-Activated protein kinase (AMPK)α, and bone morphogenetic protein (BMP) signaling, whereas the anti-angiogenic mechanistic Target of Rapamycin (mTOR) signaling is activated. *Postnatal phase*: transient activation of angiogenic signaling; the loss of elastic fibres is associated with increased proteolytic activity after IUGR. Both intrauterine and postnatal phase are related to reduced microvascular formation in lungs after IUGR and could account for the reduced lung function determined in infants born with low birth weight using Mendelian randomisation.

lular responses during angiogenesis[37]. For example, rapamycin inhibits VEGF synthesis, has antiproliferative activity, and blocks angiogenesis in vivo[37]. Likewise, it was shown that mTOR signaling regulates angiogenic sprouting[38]. Our data supports the notion that activated mTOR signaling in the intrauterine phase may interact with BMP, and disrupt angiogenic signaling after IUGR.

Proteolytic activity and disruption of elastic fiber formation may contribute to impaired lung structure and function after IUGR. Despite the postnatal catch-up of angiogenic signaling after IUGR, the number of small microvessels remains reduced. This initial paradox let us study processes independent of angionic signaling. Lung matrix is crucial in alveolarisation and serves as a scaffold that directs secondary septation and microvascular formation[39]. Experimental studies demonstrate that microvascular formation is reduced in genetically modified mice with elastin haplo-insufficiency, possibly increasing the risk for PAH[40]. Similarly, disturbed elastic fibre assembly and distribution are related to inhibition of angiogenesis in animal models of BPD[41]. BMPR2 signaling might contribute to impaired elastic fibre assembly via the BMP4-TGFβ1 pathway[40,42]. Additionally, we show a postnatal activation of metalloproteinases MMP2 an MMP2, linked to 50% reduction of elastic fibre content, persisting until P23. Increased degradation of elastic fibres might contribute to the post-IUGR alteration of lung function throughout life, including reduced FVC and increased FEV1. While our model of nutrient deprivation is highly relevant in countries, in which pregnant women are exposed to malnutrition, it does not reflect the pathomechanisms of placental insufficiency. In future studies, we recommend to confirm our findings in other models of IUGR. With our translational data from genome wide association studies, however, we aimed to correct for all causes of IUGR by defining a genetic association between LBW and lung function, independent of the cause, in a cross-sectional cohort.

Conclusion

In conclusion, our translational data show a possible association between low birth weight and lung function. Our study provides novel insight as to how IUGR disrupts lung angiogenic signaling, elastic fibre formation, and microvascular formation in a time dependent manner in an experimental rat model. Using this approach,

we identified two phases of perinatal vascular programming (Fig. 7): *first*, an intrauterine phase with inhibition of angiogenic signaling; and *second*, a postnatal phase, in which vascular signaling catches up, but lack of formation of small microvessels is related to increased proteolytic activity, and ultimately marked reduction of elastic fibre content. These data highlight how adverse intrauterine malnutrition programs the microvascular system, increasing the susceptibility to chronic lung diseases such as BPD and PAH.

Received: 30 May 2020; Accepted: 3 December 2020
Published online: 28 December 2020

References

1. Beune, I. M. *et al.* Consensus based definition of growth restriction in the newborn. *J. Pediatr.* **196**, 71-76.e71. https://doi.org/10.1016/j.jpeds.2017.12.059 (2018).
2. Pike, K., Jane Pillow, J. & Lucas, J. S. Long term respiratory consequences of intrauterine growth restriction. *Semin. Fetal Neonatal Med.* **17**, 92–98. https://doi.org/10.1016/j.siny.2012.01.003 (2012).
3. Salam, R. A., Das, J. K. & Bhutta, Z. A. Impact of intrauterine growth restriction on long-term health. *Curr. Opin. Clin. Nutr. Metab. Care* **17**, 249–254. https://doi.org/10.1097/MCO.0000000000000051 (2014).
4. Barker, D. J. Intrauterine programming of adult disease. *Mol. Med. Today* **1**, 418–423 (1995).
5. Barker, D. J. The developmental origins of chronic adult disease. *Acta Paediatr. Suppl.* **93**, 26–33 (2004).
6. Alejandre Alcazar, M. A. *et al.* Inhibition of TGF-beta signaling and decreased apoptosis in IUGR-associated lung disease in rats. *PLoS ONE* **6**, e26371. https://doi.org/10.1371/journal.pone.0026371 (2011).
7. Alejandre Alcazar, M. A. *et al.* Developmental regulation of inflammatory cytokine-mediated Stat3 signaling: The missing link between intrauterine growth restriction and pulmonary dysfunction?. *J. Mol. Med.* **90**, 945–957. https://doi.org/10.1007/s00109-012-0860-9 (2012).
8. Rona, R. J., Gulliford, M. C. & Chinn, S. Effects of prematurity and intrauterine growth on respiratory health and lung function in childhood. *BMJ* **306**, 817–820. https://doi.org/10.1136/bmj.306.6881.817 (1993).
9. Svanes, C., Omenaas, E., Heuch, J. M., Irgens, L. M. & Gulsvik, A. Birth characteristics and asthma symptoms in young adults: Results from a population-based cohort study in Norway. *Eur. Respir. J.* **12**, 1366–1370 (1998).
10. Barker, D. J. *et al.* Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. *BMJ* **303**, 671–675 (1991).
11. Zanetti, D. *et al.* Birthweight, type 2 diabetes mellitus, and cardiovascular disease: Addressing the Barker hypothesis with Mendelian randomization. *Circ. Genom. Precis. Med.* **11**, e002054. https://doi.org/10.1161/CIRCGEN.117.002054 (2018).
12. Thébaud, B. Angiogenesis in lung development, injury and repair: Implications for chronic lung disease of prematurity. *Neonatology* **91**, 291–297. https://doi.org/10.1159/000101344 (2007).
13. Thébaud, B. & Abman, S. H. Bronchopulmonary dysplasia: Where have all the vessels gone? Roles of angiogenic growth factors in chronic lung disease. *Am. J. Respir. Crit. Care Med.* **175**, 978–985. https://doi.org/10.1164/rccm.200611-1660PP (2007).
14. Sieber, C., Kopf, J., Hiepen, C. & Knaus, P. Recent advances in BMP receptor signaling. *Cytokine Growth Factor Rev.* **20**, 343–355. https://doi.org/10.1016/j.cytogfr.2009.10.007 (2009).
15. Alastalo, T. P. *et al.* Disruption of PPARgamma/beta-catenin-mediated regulation of apelin impairs BMP-induced mouse and human pulmonary arterial EC survival. *J. Clin. Investig.* **121**, 3735–3746. https://doi.org/10.1172/JCI43382 (2011).
16. Rozance, P. J. *et al.* Intrauterine growth restriction decreases pulmonary alveolar and vessel growth and causes pulmonary artery endothelial cell dysfunction in vitro in fetal sheep. *Am. J. Physiol. Lung Cell Mol. Physiol.* **301**, L860-871. https://doi.org/10.1152/ajplung.00197.2011 (2011).
17. Warrington, N. M. *et al.* Maternal and fetal genetic effects on birth weight and their relevance to cardio-metabolic risk factors. *Nat. Genet.* **51**, 804–814. https://doi.org/10.1038/s41588-019-0403-1 (2019).
18. Verbanck, M., Chen, C. Y., Neale, B. & Do, R. Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat. Genet.* **50**, 693–698. https://doi.org/10.1038/s41588-018-0099-7 (2018).
19. Burgess, S., Bowden, J., Fall, T., Ingelsson, E. & Thompson, S. G. Sensitivity analyses for robust causal inference from Mendelian randomization analyses with multiple genetic variants. *Epidemiology* **28**, 30–42. https://doi.org/10.1097/EDE.0000000000000559 (2017).
20. Rees, J. M. B., Wood, A. M. & Burgess, S. Extending the MR-Egger method for multivariable Mendelian randomization to correct for both measured and unmeasured pleiotropy. *Stat. Med.* **36**, 4705–4718. https://doi.org/10.1002/sim.7492 (2017).
21. Burgess, S. Sample size and power calculations in Mendelian randomization with a single instrumental variable and a binary outcome. *Int. J. Epidemiol.* **43**, 922–929. https://doi.org/10.1093/ije/dyu005 (2014).
22. Burgess, S. *et al.* Guidelines for performing Mendelian randomization investigations. *Wellcome Open Res.* **4**, 186. https://doi.org/10.12688/wellcomeopenres.15555.2 (2019).
23. Burgess, S. *et al.* Using published data in Mendelian randomization: A blueprint for efficient identification of causal risk factors. *Eur. J. Epidemiol.* **30**, 543–552. https://doi.org/10.1007/s10654-015-0011-z (2015).
24. Plank, C. *et al.* Intrauterine growth retardation aggravates the course of acute mesangioproliferative glomerulonephritis in the rat. *Kidney Int.* **70**, 1974–1982. https://doi.org/10.1038/sj.ki.5001966 (2006).
25. Knerr, I. *et al.* Alteration of neuronal and endothelial nitric oxide synthase and neuropeptide Y in congenital ureteropelvic junction obstruction. *Urol. Res.* **29**, 134–140 (2001).
26. Hernandez-Morera, P., Travieso-Gonzalez, C. M., Castano-Gonzalez, I., Mompeo-Corredera, B. & Ortega-Santana, F. Segmentation of elastic fibres in images of vessel wall sections stained with Weigert's resorcin-fuchsin. *Comput. Methods Programs Biomed.* **142**, 43–54. https://doi.org/10.1016/j.cmpb.2017.02.018 (2017).
27. Cottin, V. *et al.* Effect of emphysema extent on serial lung function in patients with idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **196**, 1162–1171. https://doi.org/10.1164/rccm.201612-2492OC (2017).
28. Cerveri, I. *et al.* Assessment of emphysema in COPD: A functional and radiologic study. *Chest* **125**, 1714–1718. https://doi.org/10.1378/chest.125.5.1714 (2004).
29. Kunig, A. M. *et al.* Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. *Am. J. Physiol. Lung Cell Mol. Physiol.* **289**, L529-535. https://doi.org/10.1152/ajplung.00336.2004 (2005).
30. Breslin, J. W., Pappas, P. J., Cerveira, J. J., Hobson, R. W. & Durán, W. N. VEGF increases endothelial permeability by separate signaling pathways involving ERK-1/2 and nitric oxide. *Am. J. Physiol. Heart Circ. Physiol.* **284**, H92–H100. https://doi.org/10.1152/ajpheart.00330.2002 (2003).
31. Li, Y., Sun, R., Zou, J., Ying, Y. & Luo, Z. Dual roles of the AMP-activated protein kinase pathway in angiogenesis. *Cells* https://doi.org/10.3390/cells8070752 (2019).

32. Panchision, D. M. *et al.* Sequential actions of BMP receptors control neural precursor cell production and fate. *Genes Dev.* **15**, 2094–2110. https://doi.org/10.1101/gad.894701 (2001).
33. Yang, J. *et al.* Id proteins are critical downstream effectors of BMP signaling in human pulmonary arterial smooth muscle cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* **305**, L312-321. https://doi.org/10.1152/ajplung.00054.2013 (2013).
34. Liang, S. *et al.* PDGF-BB/KLF4/VEGF signaling axis in pulmonary artery endothelial cell angiogenesis. *Cell Physiol. Biochem.* **41**, 2333–2349. https://doi.org/10.1159/000475652 (2017).
35. Visser, Y. P., Walther, F. J., Laghmani, E. H., Laarse, A. & Wagenaar, G. T. Apelin attenuates hyperoxic lung and heart injury in neonatal rats. *Am. J. Respir. Crit. Care Med.* **182**, 1239–1250. https://doi.org/10.1164/rccm.200909-1361OC (2010).
36. Watabe, T. Roles of old players in the suppression of a new player: Networks for the transcriptional control of angiogenesis. *J. Biochem.* **149**, 117–119. https://doi.org/10.1093/jb/mvq146 (2011).
37. Guba, M. *et al.* Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: Involvement of vascular endothelial growth factor. *Nat. Med.* **8**, 128–135. https://doi.org/10.1038/nm0202-128 (2002).
38. Farhan, M. A., Carmine-Simmen, K., Lewis, J. D., Moore, R. B. & Murray, A. G. Endothelial cell mTOR complex-2 regulates sprouting angiogenesiS. *PLoS ONE* **10**, e0135245. https://doi.org/10.1371/journal.pone.0135245 (2015).
39. Bourbon, J., Boucherat, O., Chailley-Heu, B. & Delacourt, C. Control mechanisms of lung alveolar development and their disorders in bronchopulmonary dysplasia. *Pediatr. Res.* **57**, 38R-46R. https://doi.org/10.1203/01.PDR.0000159630.35883.BE (2005).
40. Hilgendorff, A. *et al.* Lung matrix and vascular remodeling in mechanically ventilated elastin haploinsufficient newborn mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* **308**, L464-478. https://doi.org/10.1152/ajplung.00278.2014 (2015).
41. Pierce, R. A. *et al.* Chronic lung injury in preterm lambs: Disordered pulmonary elastin deposition. *Am. J. Physiol.* **272**, L452-460 (1997).
42. Tojais, N. F. *et al.* Codependence of bone morphogenetic protein receptor 2 and transforming growth factor-β in elastic fiber assembly and its perturbation in pulmonary arterial hypertension. *Arterioscler. Thromb. Vasc. Biol.* **37**, 1559–1569. https://doi.org/10.1161/ATVBAHA.117.309696 (2017).

Acknowledgments

Genome-wide association studies summary statistics of birth weight has been performed by the EGG Consortium (http://www.egg-consortium.org) Genome-wide association studies summary statistics of lung function (FEV1 and FVC) in the UK Biobank has been performed by Neale et al. (http://www.nealelab.is/uk-biobank/).

Author contributions

C.K.M., D.Z. and M.A.A.A. conceived and designed research; C.K.M., D.Z., C.V., M.M., and M.A.A.A. performed experiments; C.K.M., D.Z., and M.A.A.A. analyzed data; C.K.M., D.Z., C.V., L.F., M.O., U.F.M., J.D., and M.A.A.A. interpreted results of experiments; C.K.M., D.Z., and M.A.A.A. drafted manuscript; C.K.M., D.Z., C.V., L.F., M.O., U.F.M., J.D., and M.A.A.A. edited and revised manuscript. C.K.M., D.Z., C.V., L.F., M.O., U.F.M., J.D., and M.A.A.A. approved final version of manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL. This work was supported by Deutsche Forschungsgemeinschaft [AL1632/2-1 (MAAA), and by the Center for Molecular Medicine Cologne, faculty of medicine and university hospital Cologne, Germany (CMMC; MAAA).

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-020-79245-7.

Correspondence and requests for materials should be addressed to M.A.A.A.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

3. CONTRIBUTION OF COAUTHORS

This cumulative doctoral thesis is based upon the work recently published in *Scientific Reports*, under the title “Mendelian randomization and experimental IUGR reveal the adverse effect of low birth weight on lung structure and function”(1). In order to provide a clear overview of the contribution of the various coauthors to this publication, this chapter provides two clarifying tables.

Table 1: contribution of the coauthors to the article “Mendelian randomization and experimental IUGR reveal the adverse effect of low birth weight on lung structure and function”, Kuiper-Makris et al., 2020 (1).

Coauthor	***Contribution***
D. Zanetti	Mendelian Randomization studies
C. Vohlen	Introduction to experimental methods
L. Fahle	Interpretation of the data and revising manuscript
M. Müller	Immunohistochemical staining
M. Odenthal	Immunohistochemistry (anti-vWF staining)
U.Felderhoff-Müser	Interpretation of the data and revising manuscript
J. Dötsch	Interpretation of the data and revising manuscript
M.A. Alejandre Alcázar	Supervision, introduction to theoretical methods

Table 2: contribution of the coauthors to the various figures and tables in the article. Animal studies were performed by Miguel A. Alejandre Alcázar, as previously described (103, 134), in accordance with the German regulations and legal requirements and approved by the local government authorities (Regierung von Mittelfranken, AZ 621-2531.31-11/02 and AZ 621-2531.31-14/05)(222).

Figure	*Part*	*Contributing author*
1	A	Study design (C. Kuiper-Makris and M.A. Alejandre Alcázar)
	B	Scheme depicting the rat model of IUGR (C. Kuiper-Makris)
	C	Graphical presentation of body weights of rats at embryonic day 21 (E21), postnatal day 3 (P3) and P23 (C. Kuiper-Makris)
2	A	Immunohistochemical staining of lung vessels using vWF as a marker (M. Odenthal and M. Müller) and representative images (C. Kuiper-Makris)
	B	Quantification of microvessels in lungs at P3 and P23 (by C. Kuiper-Makris)
	C	Measurement of gene expression of endothelial cell markers *Cdh5* and *Pecam1* at E21, P3 and P23 using qRT-PCR (C. Kuiper-Makris)
	D	Assessment of protein abundance of the endothelial cell marker VE-Cadherin) using western blot followed by protein quantification relative to the loading control (C. Kuiper-Makris)

3	A	Measurement of gene expression of VEGF-signaling components (*Vegfa*, *Flt1*, *Flk1*) using qRT-PCR (C. Kuiper-Makris)
	B	Assessment of protein abundance of phosphorylated Erk1/2 (pErk1/2) and total Erk1/2 at E21 (C. Vohlen), P3 (C. Kuiper-Makris) and P23 (C. Kuiper-Makris) using western blot, followed by protein quantification relative to the loading control (C. Kuiper-Makris)
4	A	Measurement of gene expression of BMP-signaling components (*Bmpr1a*, *Bmpr1b*, *Bmpr2*) using qRT-PCR (C. Kuiper-Makris)
	B	Assessment of protein abundance of phosphorylated Smad1/5/8 (pSmad1/5/8) and total pSmad1 at E21, P3 and P23 using western blot, followed by protein quantification relative to the loading control (C. Kuiper-Makris)
	C	Measurement of gene expression of angiogenic and transcription factor (*Apln*, *Id1*, *Klf4*) using qRT-PCR (C. Kuiper-Makris)
	D	Assessment of Klf4 protein abundance at E21, P3 and P23 using western blot, followed by protein quantification relative to the loading control (C. Kuiper-Makris)
5	A	Assessment of protein abundance of phosphorylated AMPKα (pAMPKα) and total AMPKα at E21, P3 and P23 using western blot, followed by protein quantification relative to the loading control (C. Kuiper-Makris)
	B	Assessment of protein abundance of phosphorylated 4E-BP1 (p4E-BP1) and total 4E-BP1 at E21, P3 and P23 using western blot, followed by protein quantification relative to the loading control (C. Kuiper-Makris)
6	A	Hart's elastin stain to assess lung elastic fibers at P3 and P23; representative images (C. Kuiper-Makris)
	B	Quantification of elastic fibers at P3 and P23 (C. Kuiper-Makris)
	C	Measurement of gene expression of elastin (*Eln*) using qRT-PCR (C. Kuiper-Makris)
	D	Gelatin Zymography of lungs at P3 and P23 (C. Vohlen), followed by quantification (C. Kuiper-Makris)
7	A	Working Model by C. Kuiper-Makris and M.A. Alejandre Alcázar
Table		*Contributing author*
1		Mendelian randomization by D. Zanetti

4. DISCUSSION

The present study provides evidence for the first time, that IUGR as a result of maternal malnutrition leads to an intrauterine and postnatal dysregulation of angiogenic signaling pathways along with elevated lung proteolytic activity and a marked reduction of elastic fibers. These adverse effects of intrauterine nutrient deprivation are related to reduced formation of pulmonary microvessels. Collectively, these data offer a novel insight into the pathomechanisms by which IUGR increases the risk for CLD. To provide a link between the experimental data in the animal model and human data, we performed Mendelian Randomization in close collaboration with Dr. Daniela Zanetti (Division of Cardiovascular Medicine, Department of Medicine, Stanford University School of Medicine and Stanford Cardiovascular Institute, Stanford University, Stanford, CA, USA). Experimental and human data show that low birth weight as a proxy for IUGR not only influences the lung on a molecular and structural level, but is also associated with impaired lung function (reduced FEV1 and FVC). This possible causal association between birthweight and lung function supports the theory of pulmonary perinatal programming after IUGR. *These results are thoroughly discussed in the paper and therefore will not be part of this discussion chapter.*

4.1. The molecular link between IUGR and angiogenesis

The present study identifies two distinct phases of altered lung development: first, the prenatal phase in which growth factors are reduced, and second, the postnatal phase that shows a diminished capillary formation while the expression of growth factors in IUGR is similar to non-IUGR rats. In the prenatal phase, we have shown that two major families of growth factors are dysregulated: angiogenic signaling, including VEGF and BMP; and pathways that are central in nutrient sensing, namely the AMPKα and mTOR-signaling cascade. While the VEGF and BMP families are involved in angiogenesis and ultimately in alveolarisation (43, 46, 50, 143, 168, 178, 228, 229), they additionally regulate ECM formation. Interestingly, mTOR-signaling is essential for nutrient sensing, but also for anti-angiogenic stimuli during vessel sprouting (194, 195, 202). In the following sections, the individual signaling pathways are discussed in detail in the context of IUGR and the data of the present work.

4.1.1. The VEGF-pathway

Our study has shown that IUGR causes a transient reduction of *VegfA* expression and its receptors, intimately linked to impaired vascular formation. As stated before, VEGF induces endothelial cell proliferation, migration and sprouting as well as primitive vessel-

like tubule formation (26, 147-149). In addition, VEGF is essential for the maintenance of vessel integrity (143, 230). Studies in rodent models demonstrated that knocking out VEGFR1, VEGFR2 or VEGF-A results in embryonic lethality due to lack of vessel formation (143, 230, 231). Moreover, research has shown that the inhibition of VEGF by Su-5416, a VEGF-receptor inhibitor, impairs pulmonary angiogenesis and results in a BPD-like phenotype in a neonatal rat model (39, 232). Finally, an overexpression of VEGF also disrupts the VEGF-signaling pathway and causes a phenotype of disorganized, 'leaky' vessels, resembling pulmonary inflammatory processes similar to those seen in asthma (233, 234). These data highlight the finely-tuned VEGF pathway in the developing lung and how an imbalance of this system disrupts angiogenesis and affects quality of newly formed microvessels, and could thereby have a far-reaching influence on the pulmonary vascular bed as well as lung growth and function.

A consequence of the early dysregulation of *Vegf*(-receptor) expression is the dysregulation of the Erk1/2-signaling cascade, as demonstrated by our data. Multiple studies have shown that Erk1/2 is essential for angiogenesis. Knocking out *Erk1/2* in endothelial cells during fetal development is lethal in mice, likely related to reduced proliferation and migration of endothelial cells (162). Similarly, *in vitro* studies showed that pharmaceutical blockage of Erk1/2 reduces fetal endothelial cell proliferation and migration in cell culture (49, 161). In addition, a mouse study of hyperoxia-induced BPD has shown that acute hyperoxia transiently activates Erk1/2, whereas prolonged hyperoxia reduces Erk1/2 activation (49). Our data, combined with the existing body of knowledge for BPD models, therefore provides a possible molecular link of VEGF-signaling between IUGR and CLD.

4.1.2. The BMP-pathway

In addition to the VEGF pathway, the BMPs and their receptors are a focus of research into the pathomechanisms of chronic lung vascular diseases such as PAH (170-172). The present study provides initial evidence that a dysregulation of *Bmp*-expression and receptor activation could be involved in the pathogenesis of IUGR-associated lung disease as well. Reduced *Bmp*(-receptor) expression has been previously associated with a reduction of angiogenesis and a stiffening of the ECM, as discussed in chapter 1.3.2.. However, until now, no studies have shown an additional role for the BMP family in the predisposition for neonatal CLD.

In addition to a dysregulated expression of *Bmpr-Ia, Bmpr-Ib and Bmpr-II*, we show an increased phosphorylation of Smad1/5/8 at P3 after IUGR. This activation of BMP-

signaling might be a response to reduced vascularization during the intrauterine phase. Research has shown interactions between VEGF and BMP-2, 4 and 6, demonstrating a role for BMP as an activator of VEGF-mediated angiogenesis (229, 235, 236). It is also noteworthy that altered BMP-expression does not only affect angiogenesis, but also has a direct influence on alveolarisation: BMP has a role in the activation and proliferation of AEC II cells as well as differentiating AEC I cells, thereby contributing to lung regeneration after acute or chronic injury (168).

An important target of the BMP-pathway is apelin. Our work shows that *Apln* mRNA expression is reduced in the prenatal and early postnatal phase. This finding together with an increased expression of *Bmpr-II* on P3 possibly shows the inhibitory effect of BMP on apelin, as it was reported before (181). However, in the prenatal phase, when the BMP expression is also significantly lower than in the control group, *Apln* expression is reduced as well. While this finding does not match with the reported inhibitory properties of BMP on apelin, it is consistent with contradictive reports in literature showing an increase of apelin after stimulation with BMP-2 (237). We hypothesize that this might reflect the two-phased mechanism of prenatal and postnatal reduction of angiogenesis and alveologenesis. The present data demonstrate a marked prenatal reduction of angiogenic signaling along with reduced expression of angiogenic transcription factors such as apelin. On the other hand, the catch-up and normalization of angiogenic signaling, in particular BMPR-II, during the postnatal phase might contribute to a persistent dysregulation of angiogenic transcription factors and cause a reduction of apelin. Interestingly, a study in neonatal rats with hyperoxic lung injury has shown that apelin administration reduces hyperoxic lung injury, increasing alveolarisation and pulmonary angiogenesis compared to control groups, indicating a possible therapeutic role for apelin in hyperoxia-induced BPD and CLD (186).

In addition to the dysregulation of *Apln* expression, we have demonstrated altered *Id1* and *Klf4* mRNA expression in the prenatal phase as well. In line with the current knowledge on ID1 (chapter 1.3.2.), a reduction of *Id1* would be expected to be accompanied by a reduction of angiogenesis (187-190). The marked prenatal downregulation of *Klf4* might affect the vessel integrity as well and cause a reduction of angiogenesis (191-193). In conclusion, the dysregulation of the BMP-signaling pathway and its targets (apelin, ID1 and Klf4) as seen in our model for IUGR are likely part of a pathomechanism leading to reduced pulmonary angiogenesis and alveolarisation.

4.1.3. The mTOR- and AMPKα pathway

An interesting link between the reduced nutrient status during IUGR and dysregulated angiogenic signaling is the mTOR- and AMPKα pathway. In chapter 1.3.3., a summary of the current knowledge on this pathway highlighted a cross talk between nutrient sensing cascades and the angiogenic signaling pathways studied here (203, 204). This further underscores that angiogenesis does not only depend on cell division, migration and differentiation, but also on cellular nutrient status. In this light, our data, showing an increase of 4E-BP1 and a decrease of AMPKα at E21 during nutrient deprivation indicate a downregulation of the mTOR- and AMPK pathway, respectively. The consequences of this altered nutrient sensing machinery might be a lack of cell growth and other anabolic processes, thereby reducing the potential for angiogenesis and alveologenesis.

4.2. The structural link between IUGR and angiogenesis

IUGR adversely influences lung structure significantly in three possible ways: 1) previous research has shown that alveolarisation is reduced directly after birth, but catches up during the postnatal phase (238-240), 2) the present data shows a significantly reduced number of capillaries after the completion of alveolarisation and 3) a persisting significantly reduced number of elastic fibers linked to an increased activity of ECM-degrading metalloproteinases . This reflects the plasticity of lung development and the complexity of pulmonary development and maturation under the influence of IUGR as a prenatal hit, followed-by the postnatal hit of catch-up growth.

4.2.1. IUGR causes a persistent loss of capillaries

The present data show a persistent loss of capillaries after IUGR in a rat model. Our study focused on the elucidation of the effect of IUGR on the pulmonary vasculature along with the dynamic of the intrauterine-to-postnatal transition. The main body of current knowledge focusses on the effect of prematurity, postnatal injury, and subsequent BPD (for which IUGR is a risk factor) on pulmonary angiogenesis (241, 242). A single study inducing IUGR in sheep by placental dysfunction through temperature elevation showed a decrease of small vessels in IUGR fetuses. However, no gestational age at investigation or cut-off values for vessel quantification were described (111). The finding that IUGR results in postnatal reduction of capillaries supports the notion that IUGR is a single risk factor within a larger framework of multiple hits that collectively could lead to BPD. In addition, it would explain for the clinical observation that IUGR is associated with CLD beyond infancy and less with acute neonatal lung diseases that is independent of prematurity and BPD (136).

Interestingly, the expression of vessel markers platelet endothelial cell adhesion molecule (*Pecam1*) and vascular endothelial- Cadherin (*VE-Cadherin*) mRNA are significantly reduced prenatally. Pecam1, also known as CD31 (cluster of differentiation 31), is expressed by endothelial cells and promotes vessel formation and migration (243). Research in baboons has shown that its expression physiologically increases in the last third of gestation and is intimately linked with the migration and differentiation of pulmonary endothelial cells (244). In addition, they showed a significantly lower expression of Pecam1 after premature birth and successive respiratory therapy, in line with our finding of prenatal reduction of Pecam1 (244). VE-Cadherin (also known as Cdh5, Cadherin 5) provides the cell-cell adherence of endothelial cells and is therefore vital for the vessel integrity (245, 246). The prenatal and directly postnatal decrease of *VE-Cadherin* expression is not only in accordance with the reduction of *Pecam1* expression but also suggests that the integrity of the already formed vessels might be adversely affected. The reduced expression of vessel markers after IUGR corresponds with previous reports of reduced angiogenesis in BPD patients and animal models (41, 43, 49, 50, 241).

The significant increase of the absolute number of microvessels between P3 and P23 indicate a catch-up growth of the pulmonary vasculature, but could possible result in impaired quality of vessels. It has been demonstrated that accelerated vessel growth such as seen in tumor growth and under therapy with recombinant VEGF, induces the outgrowth of morphologically immature microvessels (50, 247). Although the current study does not investigate the functional properties of the pulmonary vessels, it gives rise to further research questions involving endothelial cell integrity and their spatial organization within the alveolar septae. In addition, dysregulated BMP-signaling after IUGR could be associated with remodeling of the vessel wall, thereby increasing the risk for PAH. Of course, this might have implications for other organs, including the cardiovascular system, as well, but that is beyond the scope of this study.

4.2.2. Lung ECM remodeling after IUGR

The present study shows a persisting marked reduction of elastic fibers that is linked to an increased activity of ECM-degrading MMPs. The scaffold of the ECM is not only vital for the development of vessels, but its components also have signaling roles for pulmonary cells. For example, research has shown that the vessel marker VE-Cadherin was significantly reduced in elastin haploinsufficient mice (213). In addition, the previous paragraphs as well as chapter 1.4.2. have shown that pathways involved in angiogenesis also exert an influence on the ECM composition, especially the BMP-pathway.

The results presented here show a (non-significant) increase of mRNA expression of the elastin gene (*Eln*). An increase of *Eln* expression has been reported in hyperoxia-induced BPD models as well (248, 249). While the deposition of elastin in the lung along with the assembly and distribution of elastic fibers is disrupted in experimental models of BPD (209), our results show an absolute reduction of elastic fibers rather than a perturbed distribution. This could indicate a different pathomechanism, possibly linked to the reduction of cellular nutrients. Indeed, there is an interaction between the mTOR-signaling pathway and elastin expression and deposition; research in a model for Williams syndrome, an elastin deficiency induced by a heterozygous loss of *Eln* causing obstructive aortopathy, has shown that elastin deficiency increases mTOR-signaling (250). To date, a reversed interaction, where decreased mTOR-signaling influences elastic fiber assembly, has not been described. However, this would provide a possible mechanism, by which a deprived nutrient status reduces elastic fiber assembly through nutrient sensing pathways (i.e. mTOR), thereby negatively influencing angiogenesis.

Another reason for the reduction of elastic fiber deposition in the lungs after IUGR could be an increase of fiber degradation. The balance between production and degradation of lung ECM is crucial for proper development and growth of the lungs. In particular MMP-2 and MMP-9 play a crucial role in tissue and vascular remodeling (205, 216, 217, 251, 252). Both MMP-2 and MMP-9 have binding capacity for TGFβ, a member of the BMP family (216). In addition, research on ovarian tumors in mice have shown that MMP-$9^{-/-}$ mice exhibit a decreased level of VEGF, associated with decreased angiogenesis and tumor growth (253). These prior studies along with our data indicate that IUGR causes an increase of MMP-activity, which may lead to ECM remodeling as indicated by a reduction of elastic fibers, negatively influencing angiogenesis.

4.3. Clinical Significance

The overall goal of the present study was to provide new insights in the pathogenesis of IUGR-associated lung disease, offering novel translational cues for patients. An improved understanding of the pathomechanisms of adverse outcomes after IUGR offers the opportunity to start ‘tweaking’ the process. The long-term mission of the present research is the elucidation of the molecular mechanisms in lungs after IUGR in order to identify novel preventive and therapeutic targets for treatment of infants born IUGR. We have shown two critical windows for the origins of CLD, and therefore windows of opportunity for intervention: first, the prenatal phase with a marked dysregulation of angiogenic signaling; and second the postnatal phase with partial catch-up of angiogenic signaling, but with significant changes of the ECM such as a reduction of elastic fibers

by 50%. One important take-home message is therefore, that not only the prenatal nutrient status, but also the postnatal nutrient status and catch-up growth play an important role in angiogenesis and the developmental origins of CLD.

Further research into the mechanism of disease is essential to define novel therapeutic targets. There are strategies to influence the major dysregulated pathways of VEGF, BMP and mTOR, but these pathways are so finely-tuned and interdependent that targeting only one pathway will unlikely offer an ultimate treatment for newborn infants. There have been multiple attempts for the treatment of BPD; given the similarity between IUGR and BPD pathology, it is likely that a prevention of IUGR-associated lung complications would also beneficially affect the outcome of BPD. Several investigations have shown promising results: induction of VEGF expression has been shown to rescue vascularization in BPD models (43, 50), MMP-9 treatment for stimulation of BMPR-II after hyperoxia improves alveolarisation (228), and the inhibition of MMPs has been shown to improve PAH (254, 255). Since the present study has proven IUGR to cause fundamental and persisting damage to the developing lung, the importance of further research into the therapeutic strategies is crucial.

4.4. Limitations

This study describes IUGR in a rat model with low-protein diet-induced IUGR. Although this is a well described and established model of IUGR caused by malnutrition (225, 226, 239, 256), it does not reflect all causes for IUGR accurately. For instance, malnutrition is not the leading cause for IUGR in European countries. As described in the introduction, a large part of IUGR in the western society is caused by placental dysfunction and inflammation. These pathologies may cause an additional 'hit' to the developing embryo, because not only nutrient status but also inflammatory mechanisms and hypoxia are at work. The present study has not covered any influences of IUGR on the immune system, although there is strong evidence suggesting a link between the BMP-pathway (TGFβ, especially) and inflammation as well as between ECM changes and inflammation, indicating IUGR as a possible trigger of chronic pulmonary inflammation.

A second, important limitation of this study is its descriptive nature. In order to provide therapeutic targets and interventional goals, a mechanistic explanation for the observed findings is required. This research, however, provides ample directions for further investigations. For example, it would be of interest to connect the nutrient sensing pathway mTOR to the angiogenic signaling pathways. This could be done by actively stimulating mTOR activity *in vitro* in endothelial cells, and measuring the expression

levels of the VEGF and BMP pathway. In addition, the connection between the mTOR pathway and ECM composition could be studied into further detail as well. In particular, mice with genetic modification in components of the mTOR-signaling cascade or pharmacological treatment of pregnant dams or newborn rats with Rapamycin would provide additional insight in the mechanisms of IUGR-associated angiogenesis and CLD. Studying these mechanisms might offer the opportunity to disentangle and balance the molecular interactions driving angiogenesis and alveolarisation in the future and offer the possibility to modify them under pathological conditions for the eventual benefit of the patient.

4.5. Conclusion

This study describes for the first time the possible molecular link between IUGR and aberrant angiogenesis, via a two-phased mechanism of prenatal and postnatal dysregulation. Our data show a significant dysregulation of the VEGF- and BMP-pathway along with nutrient-sensing mTOR pathway that is ultimately associated with aberrant angiogenesis. In addition, the present findings demonstrate increased postnatal proteolytic activity in the lung coupled with a reduction of elastic fibers by 50%. Finally, dysregulation of angiogenic and nutrient sensing signaling during the intrauterine-to-postnatal phase of lung development along with matrix remodeling after IUGR were associated with reduced number of capillaries after birth.

Collectively, these data support my hypothesis that IUGR impairs pulmonary angiogenesis, possibly through dysregulation of angiogenic and nutrient sensing signaling as well as lung matrix remodeling. These findings have resulted in a better understanding of the long-term effects of IUGR, highlighting the need for prevention and treatment of complications as well as providing possible molecular therapeutic targets.

5. SUMMARY (EN)

Intrauterine growth restriction (IUGR) has been identified as a risk factor for reduced formation of alveoli, extracellular matrix (ECM) remodelling and impaired long-term lung function, features also seen in neonatal Chronic Lung Disease (nCLD). Previous research has shown that pulmonary angiogenesis is essential for alveolarisation, and has linked impaired alveolar formation to dysregulated angiogenesis in various models for nCLD and reduced postnatal lung growth. I therefore hypothesised that IUGR and subsequent postnatal catch-up growth disrupt microvascular formation in late lung development through dysregulation of angiogenic factors, thereby affecting angiogenesis, ECM formation and ultimately alveolarisation.

Experiments were performed using frozen lungs and paraformaldehyde (PFA)-fixed lungs of a low-protein diet-induced IUGR rat model at three different time points: embryonic day 21 (E21), postnatal day 3 (P3) and P23. The mRNA and protein expression of endothelial cell markers (Pecam1 and VE-Cadherin) and angiogenic factors (VEGF-family and BMP-family signalling), as well as the nutrient-sensing mTOR and AMPKα pathways were measured in whole lung homogenates, with qRT-PCR and immunoblot, respectively. The quantity of microvessels and elastic fibres was assessed with histomorphometry. In addition, the activity of metalloproteinases was assessed with zymography.

The data demonstrate two-phased perinatal programming after IUGR. The intrauterine phase (E21) is characterized by a reduction of endothelial cell markers as well as reduced mRNA expression of angiogenic factors. Protein analysis identified reduced VEGF- and BMP-signalling, as well as an activation of anti-angiogenic mTOR effectors. In the postnatal phase (P23), the lung capillaries (<20µm) are significantly reduced, the expression of angiogenic factors and endothelial cell markers were unaffected, but Smad1/5/8 signalling and Klf4 protein mRNA expression were increased. Zymography demonstrated elevated proteolytic activity of MMP2 and MMP9, linked to a 50% reduction of lung elastic fibres.

The results provide initial evidence that IUGR reduces angiogenic signalling in the lung. Even though a catch-up growth induces a stabilisation of angiogenic markers in the postnatal lung, there is a persisting loss of capillaries and disrupted ECM formation, possibly associated with a predisposition for chronic lung diseases.

6. ZUSAMMENFASSUNG (D)

Kurzfassung der Dissertationsschrift

'Dysregulation of angiogenic signaling and reduced elastic fibers are associated with impaired formation of microvessels in rat lungs after intrauterine growth restriction.'

von Centina Kuiper-Makris

Aus der Klinik und Poliklinik für Kinder- und Jugendmedizinder Universität zu Köln
Direktor: Univ.-Prof. Dr. Jörg Dötsch

Intrauterine Wachstumsrestriktion (IUGR) ist ein Risikofaktor für verminderte Alveolenbildung, Veränderungen der extrazellulären Matrix (ECM) sowie einer Minderung der langfristigen Lungenfunktion, wie auch beschrieben in neonataler chronischer Lungenerkrankung (nCLD). Vorarbeiten haben gezeigt, dass die Gefäßbildung essentiell ist für die Alveolenbildung und dass die Störung der Alveolenbildung in verschiedenen Modellen für nCLD und reduziertes postnatales Lungenwachstum auf eine Dysregulation der Gefäßbildung zurück zu führen ist. Diese Kenntnisse führten zu der Hypothese, dass IUGR und das darauffolgende Aufholwachstum die Gefäßentwicklung in den letzten Stadien der Lungenentwicklung dysregulieren und dabei zu einem Entwicklungsstillstand der Lungen führen.
IUGR wurde in Ratten durch eine Proteinmangel-Diät (8% Casein; IUGR) der Muttertiere während der Gestation induziert; die Kontroll-Muttertiere erhielten stets Normal-Protein-Diät (17% Casein; Co). Die Lunge wurde an folgenden Zeitpunkten entnommen: embryonalen Tag 21 (E21), postnatalen Tag 1 (P1) und postnataler Tag 23 (P23), und entweder in Paraformaldehyd (PFA) fixiert oder bei -80°C eingefroren. Die mRNA und Protein Expression von Endothelzellmarkern und angiogenetische Faktoren wurde an den Lungenproben mittels qRT-PCR und Westernblot untersucht. Außerdem wurden zur Quantifizierung der Mikrogefäße und elastischen Fasern histomorphologische Untersuchung an den PFA fixierten Lungen durchgeführt.
Die erhobenen Daten zeigen eine zweiphasige Programmierung nach IUGR. Die intrauterine Phase kennzeichnet sich durch eine Minderung der Endothelzellmarker und angiogenetischen Faktoren. Proteinanalysen zeigten reduzierte VEGF- und BMP-Signalkaskaden, sowie eine Aktivierung der anti-angiogenetischen mTOR-Kaskade. In der postnatalen Phase zeigten sich die Endothelzellmarker unverändert, allerdings war die Anzahl der Kapillaren signifikant reduziert, begleitet von einer erhöhten Smad1/5/8 und Klf4 Protein Expression. Zudem zeigte sich in die Zymographie eine erhöhte Aktivität von MMP2 und MMP9, im Zusammenhang mit einer 50%-igen Reduktion der elastische Fasern.
Die Ergebnisse zeigen zum ersten Mal einen negativen Effekt von IUGR auf die pränatale Expression von angiogenetischen Faktoren und die Lungengefäßbildung. Obwohl sich während des Aufholwachstums die Expression der angiogenetischen Faktoren normalisiert, zeigt sich eine dauerhaft reduzierte Anzahl von Kapillaren und Veränderung der ECM, welche zu einer Prädisposition für CLD beitragen könnten.

7. REFERENCES

1. Kuiper-Makris C, Zanetti D, Vohlen C, Fahle L, Muller M, Odenthal M, Felderhoff-Muser U, Dotsch J, Alejandre Alcazar MA. Mendelian randomization and experimental IUGR reveal the adverse effect of low birth weight on lung structure and function. Sci Rep. 2020;10(1):22395.
2. Hsia CC, Hyde DM, Weibel ER. Lung Structure and the Intrinsic Challenges of Gas Exchange. Compr Physiol. 2016;6(2):827-95.
3. Massaro GD, Massaro D. Formation of pulmonary alveoli and gas-exchange surface area: quantitation and regulation. Annu Rev Physiol. 1996;58:73-92.
4. Weibel ER. The Pathway for Oxygen Structure and Function in the Mammalian Respiratory System. Cambridge, MA: Harvard University Press; 1984.
5. Weibel ER. Lung morphometry: the link between structure and function. Cell Tissue Res. 2017;367(3):413-26.
6. Storey WF, Staub NC. Ventilation of terminal air units. J Appl Physiol. 1962;17:391-7.
7. Crapo JD, Barry BE, Gehr P, Bachofen M, Weibel ER. Cell number and cell characteristics of the normal human lung. Am Rev Respir Dis. 1982;126(2):332-7.
8. Cochrane CG, Revak SD. Pulmonary surfactant protein B (SP-B): structure-function relationships. Science. 1991;254(5031):566-8.
9. Desai TJ, Brownfield DG, Krasnow MA. Alveolar progenitor and stem cells in lung development, renewal and cancer. Nature. 2014;507(7491):190-4.
10. Thurlbeck WM. Postnatal human lung growth. Thorax. 1982;37(8):564-71.
11. Narayanan M, Owers-Bradley J, Beardsmore CS, Mada M, Ball I, Garipov R, Panesar KS, Kuehni CE, Spycher BD, Williams SE, Silverman M. Alveolarization continues during childhood and adolescence: new evidence from helium-3 magnetic resonance. Am J Respir Crit Care Med. 2012;185(2):186-91.
12. Kajekar R. Environmental factors and developmental outcomes in the lung. Pharmacol Ther. 2007;114(2):129-45.
13. Domyan ET, Ferretti E, Throckmorton K, Mishina Y, Nicolis SK, Sun X. Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. Development. 2011;138(5):971-81.
14. Miller MF, Cohen ED, Baggs JE, Lu MM, Hogenesch JB, Morrisey EE. Wnt ligands signal in a cooperative manner to promote foregut organogenesis. Proc Natl Acad Sci U S A. 2012;109(38):15348-53.
15. Kitaoka H, Burri PH, Weibel ER. Development of the human fetal airway tree: analysis of the numerical density of airway endtips. Anat Rec. 1996;244(2):207-13.
16. Cardoso WV, Lu J. Regulation of early lung morphogenesis: questions, facts and controversies. Development. 2006;133(9):1611-24.
17. deMello DE, Sawyer D, Galvin N, Reid LM. Early fetal development of lung vasculature. Am J Respir Cell Mol Biol. 1997;16(5):568-81.
18. Howatt WF, Avery ME, Humphreys PW, Normand IC, Reid L, Strang LB. Factors affecting pulmonary surface properties in the foetal lamb. Clin Sci. 1965;29(2):239-48.
19. Schittny JC. Development of the lung. Cell Tissue Res. 2017;367(3):427-44.
20. Zeltner TB, Burri PH. The postnatal development and growth of the human lung. II. Morphology. Respir Physiol. 1987;67(3):269-82.
21. Zeltner TB, Caduff JH, Gehr P, Pfenninger J, Burri PH. The postnatal development and growth of the human lung. I. Morphometry. Respir Physiol. 1987;67(3):247-67.
22. Burri PH. Structural aspects of postnatal lung development - alveolar formation and growth. Biol Neonate. 2006;89(4):313-22.
23. Lindahl P, Karlsson L, Hellstrom M, Gebre-Medhin S, Willetts K, Heath JK, Betsholtz C. Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. Development. 1997;124(20):3943-53.
24. Margraf LR, Tomashefski JF, Jr., Bruce MC, Dahms BB. Morphometric analysis of the lung in bronchopulmonary dysplasia. Am Rev Respir Dis. 1991;143(2):391-400.
25. Pierce RA, Albertine KH, Starcher BC, Bohnsack JF, Carlton DP, Bland RD. Chronic lung injury in preterm lambs: disordered pulmonary elastin deposition. Am J Physiol. 1997;272(3 Pt 1):L452-60.
26. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996;380(6573):435-9.
27. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science. 1997;277(5322):55-60.
28. Morrisey EE, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell. 2010;18(1):8-23.
29. Gebb SA, Shannon JM. Tissue interactions mediate early events in pulmonary vasculogenesis. Dev Dyn. 2000;217(2):159-69.

30. Parera MC, van Dooren M, van Kempen M, de Krijger R, Grosveld F, Tibboel D, Rottier R. Distal angiogenesis: a new concept for lung vascular morphogenesis. Am J Physiol Lung Cell Mol Physiol. 2005;288(1):L141-9.
31. Kaner RJ, Crystal RG. Compartmentalization of vascular endothelial growth factor to the epithelial surface of the human lung. Mol Med. 2001;7(4):240-6.
32. Bhatt AJ, Amin SB, Chess PR, Watkins RH, Maniscalco WM. Expression of vascular endothelial growth factor and Flk-1 in developing and glucocorticoid-treated mouse lung. Pediatr Res. 2000;47(5):606-13.
33. Hislop A. Developmental biology of the pulmonary circulation. Paediatr Respir Rev. 2005;6(1):35-43.
34. Algire GH. Vascular reactions of normal and malignant tissues in vivo. VII. Observations on vascular reactions in destruction of tumor homografts. J Natl Cancer Inst. 1954;15(3):483-91.
35. Djonov V, Baum O, Burri PH. Vascular remodeling by intussusceptive angiogenesis. Cell Tissue Res. 2003;314(1):107-17.
36. Caduff JH, Fischer LC, Burri PH. Scanning electron microscope study of the developing microvasculature in the postnatal rat lung. Anat Rec. 1986;216(2):154-64.
37. Cagle PT, Thurlbeck WM. Postpneumonectomy compensatory lung growth. Am Rev Respir Dis. 1988;138(5):1314-26.
38. Ghammad K, Fievez P, Remy P. Compensatory Lung Growth after Pneumonectomy: Case Report. Acta Chir Belg. 2015;115(3):234-6.
39. Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF, Abman SH. Inhibition of angiogenesis decreases alveolarization in the developing rat lung. Am J Physiol Lung Cell Mol Physiol. 2000;279(3):L600-7.
40. Mammoto A, Mammoto T. Vascular Niche in Lung Alveolar Development, Homeostasis, and Regeneration. Front Bioeng Biotechnol. 2019;7:318.
41. Thebaud B, Abman SH. Bronchopulmonary dysplasia: where have all the vessels gone? Roles of angiogenic growth factors in chronic lung disease. Am J Respir Crit Care Med. 2007;175(10):978-85.
42. Hislop AA. Airway and blood vessel interaction during lung development. J Anat. 2002;201(4):325-34.
43. Kunig AM, Balasubramaniam V, Markham NE, Morgan D, Montgomery G, Grover TR, Abman SH. Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. Am J Physiol Lung Cell Mol Physiol. 2005;289(4):L529-35.
44. Yamamoto H, Yun EJ, Gerber HP, Ferrara N, Whitsett JA, Vu TH. Epithelial-vascular cross talk mediated by VEGF-A and HGF signaling directs primary septae formation during distal lung morphogenesis. Dev Biol. 2007;308(1):44-53.
45. Robinet A, Fahem A, Cauchard JH, Huet E, Vincent L, Lorimier S, Antonicelli F, Soria C, Crepin M, Hornebeck W, Bellon G. Elastin-derived peptides enhance angiogenesis by promoting endothelial cell migration and tubulogenesis through upregulation of MT1-MMP. J Cell Sci. 2005;118(Pt 2):343-56.
46. Yun EJ, Lorizio W, Seedorf G, Abman SH, Vu TH. VEGF and endothelium-derived retinoic acid regulate lung vascular and alveolar development. Am J Physiol Lung Cell Mol Physiol. 2016;310(4):L287-98.
47. Albertine KH, Jones GP, Starcher BC, Bohnsack JF, Davis PL, Cho SC, Carlton DP, Bland RD. Chronic lung injury in preterm lambs. Disordered respiratory tract development. Am J Respir Crit Care Med. 1999;159(3):945-58.
48. Fracica PJ, Knapp MJ, Piantadosi CA, Takeda K, Fulkerson WJ, Coleman RE, Wolfe WG, Crapo JD. Responses of baboons to prolonged hyperoxia: physiology and qualitative pathology. J Appl Physiol (1985). 1991;71(6):2352-62.
49. Menon RT, Shrestha AK, Barrios R, Shivanna B. Hyperoxia Disrupts Extracellular Signal-Regulated Kinases 1/2-Induced Angiogenesis in the Developing Lungs. Int J Mol Sci. 2018;19(5).
50. Thebaud B, Ladha F, Michelakis ED, Sawicka M, Thurston G, Eaton F, Hashimoto K, Harry G, Haromy A, Korbutt G, Archer SL. Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. Circulation. 2005;112(16):2477-86.
51. Chabra S, Strandjord T, Peeples E. Prolonged non-invasive ventilation in extremely low birth weight preterm infants is associated with bronchopulmonary dysplasia. J Neonatal Perinatal Med. 2019;12(3):249-53.
52. Ho JJ, Subramaniam P, Davis PG. Continuous positive airway pressure (CPAP) for respiratory distress in preterm infants. Cochrane Database Syst Rev. 2020;10:CD002271.
53. Isayama T, Iwami H, McDonald S, Beyene J. Association of Noninvasive Ventilation Strategies With Mortality and Bronchopulmonary Dysplasia Among Preterm Infants: A Systematic Review and Meta-analysis. JAMA. 2016;316(6):611-24.
54. Backes CH, Markham K, Moorehead P, Cordero L, Nankervis CA, Giannone PJ. Maternal preeclampsia and neonatal outcomes. J Pregnancy. 2011;2011:214365.
55. Lau J, Magee F, Qiu Z, Hoube J, Von Dadelszen P, Lee SK. Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than chorioamnionitis displaying a maternal inflammatory response only. Am J Obstet Gynecol. 2005;193(3 Pt 1):708-13.

56. Edwards CA, Osman LM, Godden DJ, Campbell DM, Douglas JG. Relationship between birth weight and adult lung function: controlling for maternal factors. Thorax. 2003;58(12):1061-5.
57. Harpsoe MC, Basit S, Bager P, Wohlfahrt J, Benn CS, Nohr EA, Linneberg A, Jess T. Maternal obesity, gestational weight gain, and risk of asthma and atopic disease in offspring: a study within the Danish National Birth Cohort. J Allergy Clin Immunol. 2013;131(4):1033-40.
58. Torrance HL, Voorbij HA, Wijnberger LD, van Bel F, Visser GH. Lung maturation in small for gestational age fetuses from pregnancies complicated by placental insufficiency or maternal hypertension. Early Hum Dev. 2008;84(7):465-9.
59. Whitsett JA, Wert SE, Trapnell BC. Genetic disorders influencing lung formation and function at birth. Hum Mol Genet. 2004;13 Spec No 2:R207-15.
60. Plosa E, Guttentag SH. 42 - Lung Development. In: Gleason CA, Juul SE, editors. Avery's Diseases of the Newborn (Tenth Edition). Philadelphia: Elsevier; 2018. p. 586-99.e2.
61. Pfister RE, Ramsden CA, Neil HL, Kyriakides MA, Berger PJ. Volume and secretion rate of lung liquid in the final days of gestation and labour in the fetal sheep. J Physiol. 2001;535(Pt 3):889-99.
62. Cabral JE, Belik J. Persistent pulmonary hypertension of the newborn: recent advances in pathophysiology and treatment. J Pediatr (Rio J). 2013;89(3):226-42.
63. The prevention of perinatal mortality and morbidity. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser. 1970;457:1-60.
64. Moore TA, Wilson ME, Schmid KK, Anderson-Berry A, French JA, Berger AM. Relations between feeding intolerance and stress biomarkers in preterm infants. J Pediatr Gastroenterol Nutr. 2013;57(3):356-62.
65. Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, Hale EC, Newman NS, Schibler K, Carlo WA, Kennedy KA, Poindexter BB, Finer NN, Ehrenkranz RA, Duara S, Sanchez PJ, O'Shea TM, Goldberg RN, Van Meurs KP, Faix RG, Phelps DL, Frantz ID, 3rd, Watterberg KL, Saha S, Das A, Higgins RD, Eunice Kennedy Shriver National Institute of Child H, Human Development Neonatal Research N. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. Pediatrics. 2010;126(3):443-56.
66. Kotecha SJ, Edwards MO, Watkins WJ, Henderson AJ, Paranjothy S, Dunstan FD, Kotecha S. Effect of preterm birth on later FEV1: a systematic review and meta-analysis. Thorax. 2013;68(8):760-6.
67. Gurakan B, Tarcan A, Arda IS, Coskun M. Persistent pulmonary interstitial emphysema in an unventilated neonate. Pediatr Pulmonol. 2002;34(5):409-11.
68. Yu VY, Wong PY, Bajuk B, Szymonowicz W. Pulmonary interstitial emphysema in infants less than 1000 g at birth. Aust Paediatr J. 1986;22(3):189-92.
69. He H, Butz A, Keet CA, Minkovitz CS, Hong X, Caruso DM, Pearson C, Cohen RT, Wills-Karp M, Zuckerman BS, Hughes ME, Wang X. Preterm Birth with Childhood Asthma: The Role of Degree of Prematurity and Asthma Definitions. Am J Respir Crit Care Med. 2015;192(4):520-3.
70. Rona RJ, Gulliford MC, Chinn S. Effects of prematurity and intrauterine growth on respiratory health and lung function in childhood. BMJ. 1993;306(6881):817-20.
71. Svanes C, Omenaas E, Heuch JM, Irgens LM, Gulsvik A. Birth characteristics and asthma symptoms in young adults: results from a population-based cohort study in Norway. Eur Respir J. 1998;12(6):1366-70.
72. Arjaans S, Zwart EAH, Ploegstra MJ, Bos AF, Kooi EMW, Hillege HL, Berger RMF. Identification of gaps in the current knowledge on pulmonary hypertension in extremely preterm infants: A systematic review and meta-analysis. Paediatr Perinat Epidemiol. 2018;32(3):258-67.
73. Mirza H, Ziegler J, Ford S, Padbury J, Tucker R, Laptook A. Pulmonary hypertension in preterm infants: prevalence and association with bronchopulmonary dysplasia. J Pediatr. 2014;165(5):909-14 e1.
74. Northway WH, Jr. Observations on bronchopulmonary dysplasia. J Pediatr. 1979;95(5 Pt 2):815-8.
75. Northway WH, Jr., Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med. 1967;276(7):357-68.
76. Jobe AJ. The new BPD: an arrest of lung development. Pediatr Res. 1999;46(6):641-3.
77. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001;163(7):1723-9.
78. Shennan AT, Dunn MS, Ohlsson A, Lennox K, Hoskins EM. Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period. Pediatrics. 1988;82(4):527-32.
79. Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Hum Pathol. 1998;29(7):710-7.
80. Hwang JS, Rehan VK. Recent Advances in Bronchopulmonary Dysplasia: Pathophysiology, Prevention, and Treatment. Lung. 2018;196(2):129-38.
81. Jensen EA, Schmidt B. Epidemiology of bronchopulmonary dysplasia. Birth Defects Res A Clin Mol Teratol. 2014;100(3):145-57.
82. Zeitlin J, Draper ES, Kollee L, Milligan D, Boerch K, Agostino R, Gortner L, Van Reempts P, Chabernaud JL, Gadzinowski J, Breart G, Papiernik E, group Mr. Differences in rates and short-term outcome of live births before 32 weeks of gestation in Europe in 2003: results from the MOSAIC cohort. Pediatrics. 2008;121(4):e936-44.

83. Siffel C, Kistler KD, Lewis JFM, Sarda SP. Global incidence of bronchopulmonary dysplasia among extremely preterm infants: a systematic literature review. J Matern Fetal Neonatal Med. 2019:1-11.
84. Davidson LM, Berkelhamer SK. Bronchopulmonary Dysplasia: Chronic Lung Disease of Infancy and Long-Term Pulmonary Outcomes. J Clin Med. 2017;6(1).
85. Perez Tarazona S, Solano Galan P, Bartoll Alguacil E, Alfonso Diego J. Bronchopulmonary dysplasia as a risk factor for asthma in school children and adolescents: A systematic review. Allergol Immunopathol (Madr). 2018;46(1):87-98.
86. Goss KN, Austin ED, Battiola TJ, Tepper RS, Lahm T. Novel early life risk factors for adult pulmonary hypertension. Pulm Circ. 2019;9(2):2045894019845615.
87. Wigglesworth JS. Foetal growth retardation. Br Med Bull. 1966;22(1):13-5.
88. Beune IM, Bloomfield FH, Ganzevoort W, Embleton ND, Rozance PJ, van Wassenaer-Leemhuis AG, Wynia K, Gordijn SJ. Consensus Based Definition of Growth Restriction in the Newborn. J Pediatr. 2018;196:71-6 e1.
89. Steiner M, Pomerance W. Studies on prematurity. II. Influence of fetal maturity on fatality rate. Pediatrics. 1950;6(6):872-77.
90. Taback M. Birth weight and length of gestation with relation to prematurity. J Am Med Assoc. 1951;146(10):897-901.
91. Gordijn SJ, Beune IM, Ganzevoort W. Building consensus and standards in fetal growth restriction studies. Best Pract Res Clin Obstet Gynaecol. 2018;49:117-26.
92. Kesavan K, Devaskar SU. Intrauterine Growth Restriction: Postnatal Monitoring and Outcomes. Pediatr Clin North Am. 2019;66(2):403-23.
93. Lubchenco LO, Hansman C, Dressler M, Boyd E. Intrauterine Growth as Estimated from Liveborn Birth-Weight Data at 24 to 42 Weeks of Gestation. Pediatrics. 1963;32:793-800.
94. Danaei G, Andrews KG, Sudfeld CR, Fink G, McCoy DC, Peet E, Sania A, Smith Fawzi MC, Ezzati M, Fawzi WW. Risk Factors for Childhood Stunting in 137 Developing Countries: A Comparative Risk Assessment Analysis at Global, Regional, and Country Levels. PLoS Med. 2016;13(11):e1002164.
95. Sharma D, Shastri S, Farahbakhsh N, Sharma P. Intrauterine growth restriction - part 1. J Matern Fetal Neonatal Med. 2016;29(24):3977-87.
96. Rosenberg A. The IUGR newborn. Semin Perinatol. 2008;32(3):219-24.
97. Carlson DE. Maternal diseases associated with intrauterine growth retardation. Semin Perinatol. 1988;12(1):17-22.
98. Smith CA. Effects of maternal under nutrition upon the newborn infant in Holland (1944-1945). J Pediatr. 1947;30(3):229-43.
99. Chaddha V, Viero S, Huppertz B, Kingdom J. Developmental biology of the placenta and the origins of placental insufficiency. Semin Fetal Neonatal Med. 2004;9(5):357-69.
100. Hendrix N, Berghella V. Non-placental causes of intrauterine growth restriction. Semin Perinatol. 2008;32(3):161-5.
101. Adams Waldorf KM, McAdams RM. Influence of infection during pregnancy on fetal development. Reproduction. 2013;146(5):R151-62.
102. Ziegler EE. Nutrient Needs for Catch-Up Growth in Low-Birthweight Infants. Nestle Nutr Inst Workshop Ser. 2015;81:135-43.
103. McIntire DD, Bloom SL, Casey BM, Leveno KJ. Birth weight in relation to morbidity and mortality among newborn infants. N Engl J Med. 1999;340(16):1234-8.
104. Abbas G, Shah S, Hanif M, Shah A, Rehman AU, Tahir S, Nayab K, Asghar A. The frequency of pulmonary hypertension in newborn with intrauterine growth restriction. Sci Rep. 2020;10(1):8064.
105. Inacio QAS, Araujo Junior E, Nardozza LMM, Petrini CG, Campos VP, Peixoto AB. Perinatal Outcomes of Fetuses with Early Growth Restriction, Late Growth Restriction, Small for Gestational Age, and Adequate for Gestational Age. Rev Bras Ginecol Obstet. 2019;41(12):688-96.
106. Fekete M, Nemeth A. Neonatal pulmonary haemorrhage, birthweight, gestational age and intrauterine growth. Acta Paediatr Hung. 1985;26(1):65-73.
107. Regev RH, Reichman B. Prematurity and intrauterine growth retardation--double jeopardy? Clin Perinatol. 2004;31(3):453-73.
108. Alejandre Alcazar MA, Morty RE, Lendzian L, Vohlen C, Oestreicher I, Plank C, Schneider H, Dotsch J. Inhibition of TGF-beta signaling and decreased apoptosis in IUGR-associated lung disease in rats. PLoS One. 2011;6(10):e26371.
109. Maritz GS, Cock ML, Louey S, Joyce BJ, Albuquerque CA, Harding R. Effects of fetal growth restriction on lung development before and after birth: a morphometric analysis. Pediatr Pulmonol. 2001;32(3):201-10.
110. Maritz GS, Cock ML, Louey S, Suzuki K, Harding R. Fetal growth restriction has long-term effects on postnatal lung structure in sheep. Pediatr Res. 2004;55(2):287-95.
111. Rozance PJ, Seedorf GJ, Brown A, Roe G, O'Meara MC, Gien J, Tang JR, Abman SH. Intrauterine growth restriction decreases pulmonary alveolar and vessel growth and causes pulmonary artery endothelial cell dysfunction in vitro in fetal sheep. Am J Physiol Lung Cell Mol Physiol. 2011;301(6):L860-71.
112. Barker DJ. Intrauterine programming of adult disease. Mol Med Today. 1995;1(9):418-23.
113. Barker DJ. The developmental origins of chronic adult disease. Acta Paediatr Suppl. 2004;93(446):26-33.

114. Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia. 1993;36(1):62-7.
115. Crume TL, Scherzinger A, Stamm E, McDuffie R, Bischoff KJ, Hamman RF, Dabelea D. The long-term impact of intrauterine growth restriction in a diverse U.S. cohort of children: the EPOCH study. Obesity (Silver Spring). 2014;22(2):608-15.
116. Laitinen J, Pietilainen K, Wadsworth M, Sovio U, Jarvelin MR. Predictors of abdominal obesity among 31-y-old men and women born in Northern Finland in 1966. Eur J Clin Nutr. 2004;58(1):180-90.
117. Mohan R, Baumann D, Alejandro EU. Fetal undernutrition, placental insufficiency, and pancreatic beta-cell development programming in utero. Am J Physiol Regul Integr Comp Physiol. 2018;315(5):R867-R78.
118. Newsome CA, Shiell AW, Fall CH, Phillips DI, Shier R, Law CM. Is birth weight related to later glucose and insulin metabolism?--A systematic review. Diabet Med. 2003;20(5):339-48.
119. Ravelli AC, van Der Meulen JH, Osmond C, Barker DJ, Bleker OP. Obesity at the age of 50 y in men and women exposed to famine prenatally. Am J Clin Nutr. 1999;70(5):811-6.
120. Simmons RA, Templeton LJ, Gertz SJ. Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. Diabetes. 2001;50(10):2279-86.
121. Hales CN, Barker DJ. The thrifty phenotype hypothesis. Br Med Bull. 2001;60:5-20.
122. Dunlop K, Cedrone M, Staples JF, Regnault TR. Altered fetal skeletal muscle nutrient metabolism following an adverse in utero environment and the modulation of later life insulin sensitivity. Nutrients. 2015;7(2):1202-16.
123. Ibanez L, Potau N, Marcos MV, de Zegher F. Exaggerated adrenarche and hyperinsulinism in adolescent girls born small for gestational age. J Clin Endocrinol Metab. 1999;84(12):4739-41.
124. Invitti C, Gilardini L, Mazzilli G, Sartorio A, Viberti GC. -to: Ong KK, Petry CJ, Emmett PM et al.; ALSPAC study team (2004) Insulin sensitivity and secretion in normal children related to size at birth, postnatal growth, and plasma insulin-like growth factor-I levels. Diabetologia 47:1064-1070. Diabetologia. 2004;47(11):2044-5; author reply 6.
125. Muhlhausler BS, Duffield JA, Ozanne SE, Pilgrim C, Turner N, Morrison JL, McMillen IC. The transition from fetal growth restriction to accelerated postnatal growth: a potential role for insulin signalling in skeletal muscle. J Physiol. 2009;587(Pt 17):4199-211.
126. Gluckman PD, Hanson MA. Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. Pediatr Res. 2004;56(3):311-7.
127. Nederhof E, Schmidt MV. Mismatch or cumulative stress: toward an integrated hypothesis of programming effects. Physiol Behav. 2012;106(5):691-700.
128. Longo S, Bollani L, Decembrino L, Di Comite A, Angelini M, Stronati M. Short-term and long-term sequelae in intrauterine growth retardation (IUGR). J Matern Fetal Neonatal Med. 2013;26(3):222-5.
129. Ravelli GP, Stein ZA, Susser MW. Obesity in young men after famine exposure in utero and early infancy. N Engl J Med. 1976;295(7):349-53.
130. Uemura O, Ishikura K, Kaneko T, Hirano D, Hamasaki Y, Ogura M, Mikami N, Gotoh Y, Sahashi T, Fujita N, Yamamoto M, Hibino S, Nakano M, Wakano Y, Honda M. Perinatal factors contributing to chronic kidney disease in a cohort of Japanese children with very low birth weight. Pediatr Nephrol. 2020.
131. Coupe B, Grit I, Hulin P, Randuineau G, Parnet P. Postnatal growth after intrauterine growth restriction alters central leptin signal and energy homeostasis. PLoS One. 2012;7(1):e30616.
132. Lim K, Armitage JA, Stefanidis A, Oldfield BJ, Black MJ. IUGR in the absence of postnatal "catch-up" growth leads to improved whole body insulin sensitivity in rat offspring. Pediatr Res. 2011;70(4):339-44.
133. Alcazar MA, Dinger K, Rother E, Ostreicher I, Vohlen C, Plank C, Dotsch J. Prevention of early postnatal hyperalimentation protects against activation of transforming growth factor-beta/bone morphogenetic protein and interleukin-6 signaling in rat lungs after intrauterine growth restriction. J Nutr. 2014;144(12):1943-51.
134. Soudee S, Vuillemin L, Alberti C, Mohamed D, Becquet O, Farnoux C, Biran V, Baud O. Fetal growth restriction is worse than extreme prematurity for the developing lung. Neonatology. 2014;106(4):304-10.
135. Greenough A, Yuksel B, Cheeseman P. Effect of in utero growth retardation on lung function at follow-up of prematurely born infants. Eur Respir J. 2004;24(5):731-3.
136. Ronkainen E, Dunder T, Kaukola T, Marttila R, Hallman M. Intrauterine growth restriction predicts lower lung function at school age in children born very preterm. Arch Dis Child Fetal Neonatal Ed. 2016;101(5):F412-7.
137. Cai Y, Shaheen SO, Hardy R, Kuh D, Hansell AL. Birth weight, early childhood growth and lung function in middle to early old age: 1946 British birth cohort. Thorax. 2016;71(10):916-22.
138. Roth-Kleiner M, Post M. Genetic control of lung development. Biol Neonate. 2003;84(1):83-8.
139. Alejandre Alcazar MA, Ostreicher I, Appel S, Rother E, Vohlen C, Plank C, Dotsch J. Developmental regulation of inflammatory cytokine-mediated Stat3 signaling: the missing link between intrauterine growth restriction and pulmonary dysfunction? J Mol Med (Berl). 2012;90(8):945-57.
140. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med. 1995;332(3):133-8.

141. Basson MA. Signaling in cell differentiation and morphogenesis. Cold Spring Harb Perspect Biol. 2012;4(6).
142. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol. 2007;8(6):464-78.
143. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol. 1991;5(12):1806-14.
144. Celec P, Yonemitsu Y. Vascular endothelial growth factor - basic science and its clinical implications. Pathophysiology. 2004;11(2):69-75.
145. Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML, Rossant J. flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. Development. 1993;118(2):489-98.
146. Brown KR, England KM, Goss KL, Snyder JM, Acarregui MJ. VEGF induces airway epithelial cell proliferation in human fetal lung in vitro. Am J Physiol Lung Cell Mol Physiol. 2001;281(4):L1001-10.
147. Breier G, Albrecht U, Sterrer S, Risau W. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development. 1992;114(2):521-32.
148. Kearney JB, Ambler CA, Monaco KA, Johnson N, Rapoport RG, Bautch VL. Vascular endothelial growth factor receptor Flt-1 negatively regulates developmental blood vessel formation by modulating endothelial cell division. Blood. 2002;99(7):2397-407.
149. Soga N, Connolly JO, Chellaiah M, Kawamura J, Hruska KA. Rac regulates vascular endothelial growth factor stimulated motility. Cell Commun Adhes. 2001;8(1):1-13.
150. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011;473(7347):298-307.
151. Kelly BD, Hackett SF, Hirota K, Oshima Y, Cai Z, Berg-Dixon S, Rowan A, Yan Z, Campochiaro PA, Semenza GL. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circ Res. 2003;93(11):1074-81.
152. Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber HP, Ferrara N, Johnson RS. Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. Cancer Cell. 2004;6(5):485-95.
153. Vadivel A, Alphonse RS, Etches N, van Haaften T, Collins JJ, O'Reilly M, Eaton F, Thebaud B. Hypoxia-inducible factors promote alveolar development and regeneration. Am J Respir Cell Mol Biol. 2014;50(1):96-105.
154. Yamakawa M, Liu LX, Date T, Belanger AJ, Vincent KA, Akita GY, Kuriyama T, Cheng SH, Gregory RJ, Jiang C. Hypoxia-inducible factor-1 mediates activation of cultured vascular endothelial cells by inducing multiple angiogenic factors. Circ Res. 2003;93(7):664-73.
155. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science. 2001;294(5545):1337-40.
156. Hosford GE, Olson DM. Effects of hyperoxia on VEGF, its receptors, and HIF-2alpha in the newborn rat lung. Am J Physiol Lung Cell Mol Physiol. 2003;285(1):L161-8.
157. Tsao PN, Wei SC. Prenatal hypoxia downregulates the expression of pulmonary vascular endothelial growth factor and its receptors in fetal mice. Neonatology. 2013;103(4):300-7.
158. Dor Y, Keshet E. Ischemia-driven angiogenesis. Trends Cardiovasc Med. 1997;7(8):289-94.
159. Tang JR, Markham NE, Lin YJ, McMurtry IF, Maxey A, Kinsella JP, Abman SH. Inhaled nitric oxide attenuates pulmonary hypertension and improves lung growth in infant rats after neonatal treatment with a VEGF receptor inhibitor. Am J Physiol Lung Cell Mol Physiol. 2004;287(2):L344-51.
160. Fish JE, Cantu Gutierrez M, Dang LT, Khyzha N, Chen Z, Veitch S, Cheng HS, Khor M, Antounians L, Njock MS, Boudreau E, Herman AM, Rhyner AM, Ruiz OE, Eisenhoffer GT, Medina-Rivera A, Wilson MD, Wythe JD. Dynamic regulation of VEGF-inducible genes by an ERK/ERG/p300 transcriptional network. Development. 2017;144(13):2428-44.
161. Mavria G, Vercoulen Y, Yeo M, Paterson H, Karasarides M, Marais R, Bird D, Marshall CJ. ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. Cancer Cell. 2006;9(1):33-44.
162. Srinivasan R, Zabuawala T, Huang H, Zhang J, Gulati P, Fernandez S, Karlo JC, Landreth GE, Leone G, Ostrowski MC. Erk1 and Erk2 regulate endothelial cell proliferation and migration during mouse embryonic angiogenesis. PLoS One. 2009;4(12):e8283.
163. Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, Maksimovic-Ivanic D, Stivala F, Mazzarino MC, Donia M, Fagone P, Malaponte G, Nicoletti F, Libra M, Milella M, Tafuri A, Bonati A, Basecke J, Cocco L, Evangelisti C, Martelli AM, Montalto G, Cervello M, McCubrey JA. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. Aging (Albany NY). 2011;3(3):192-222.
164. Yoon S, Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors. 2006;24(1):21-44.
165. Cobb MH, Boulton TG, Robbins DJ. Extracellular signal-regulated kinases: ERKs in progress. Cell Regul. 1991;2(12):965-78.
166. Roskoski R, Jr. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol Res. 2012;66(2):105-43.

167. Roberts JR, Perkins GD, Fujisawa T, Pettigrew KA, Gao F, Ahmed A, Thickett DR. Vascular endothelial growth factor promotes physical wound repair and is anti-apoptotic in primary distal lung epithelial and A549 cells. Crit Care Med. 2007;35(9):2164-70.
168. Chung MI, Bujnis M, Barkauskas CE, Kobayashi Y, Hogan BLM. Niche-mediated BMP/SMAD signaling regulates lung alveolar stem cell proliferation and differentiation. Development. 2018;145(9).
169. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet. 2000;67(3):737-44.
170. Orriols M, Gomez-Puerto MC, Ten Dijke P. BMP type II receptor as a therapeutic target in pulmonary arterial hypertension. Cell Mol Life Sci. 2017;74(16):2979-95.
171. Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JS, Egan J, Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, Nichols WC. Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. J Med Genet. 2000;37(10):741-5.
172. Beppu H, Ichinose F, Kawai N, Jones RC, Yu PB, Zapol WM, Miyazono K, Li E, Bloch KD. BMPR-II heterozygous mice have mild pulmonary hypertension and an impaired pulmonary vascular remodeling response to prolonged hypoxia. Am J Physiol Lung Cell Mol Physiol. 2004;287(6):L1241-7.
173. Dhore CR, Cleutjens JP, Lutgens E, Cleutjens KB, Geusens PP, Kitslaar PJ, Tordoir JH, Spronk HM, Vermeer C, Daemen MJ. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. Arterioscler Thromb Vasc Biol. 2001;21(12):1998-2003.
174. Ye L, Jiang WG. Bone morphogenetic proteins in tumour associated angiogenesis and implication in cancer therapies. Cancer Lett. 2016;380(2):586-97.
175. Jeong JW, Kim M, Lee J, Lee HK, Ko Y, Kim H, Fang S. ID1-Mediated BMP Signaling Pathway Potentiates Glucagon-Like Peptide-1 Secretion in Response to Nutrient Replenishment. Int J Mol Sci. 2020;21(11).
176. Zhou G, Sun YG, Wang HB, Wang WQ, Wang XW, Fang DC. Acid and bile salt up-regulate BMP4 expression in human esophageal epithelium cells. Scand J Gastroenterol. 2009;44(8):926-32.
177. Miyazono K, Kamiya Y, Morikawa M. Bone morphogenetic protein receptors and signal transduction. J Biochem. 2010;147(1):35-51.
178. Zhou Q, Heinke J, Vargas A, Winnik S, Krauss T, Bode C, Patterson C, Moser M. ERK signaling is a central regulator for BMP-4 dependent capillary sprouting. Cardiovasc Res. 2007;76(3):390-9.
179. Simic P, Vukicevic S. Bone morphogenetic proteins: from developmental signals to tissue regeneration. Conference on bone morphogenetic proteins. EMBO Rep. 2007;8(4):327-31.
180. He L, Xu J, Chen L, Li L. Apelin/APJ signaling in hypoxia-related diseases. Clin Chim Acta. 2015;451(Pt B):191-8.
181. Poirier O, Ciumas M, Eyries M, Montagne K, Nadaud S, Soubrier F. Inhibition of apelin expression by BMP signaling in endothelial cells. Am J Physiol Cell Physiol. 2012;303(11):C1139-45.
182. Brash L, Barnes GD, Brewis MJ, Church AC, Gibbs SJ, Howard L, Jayasekera G, Johnson MK, McGlinchey N, Onorato J, Simpson J, Stirrat C, Thomson S, Watson G, Wilkins MR, Xu C, Welsh DJ, Newby DE, Peacock AJ. Short-Term Hemodynamic Effects of Apelin in Patients With Pulmonary Arterial Hypertension. JACC Basic Transl Sci. 2018;3(2):176-86.
183. Falcao-Pires I, Goncalves N, Henriques-Coelho T, Moreira-Goncalves D, Roncon-Albuquerque R, Jr., Leite-Moreira AF. Apelin decreases myocardial injury and improves right ventricular function in monocrotaline-induced pulmonary hypertension. Am J Physiol Heart Circ Physiol. 2009;296(6):H2007-14.
184. Helker CS, Eberlein J, Wilhelm K, Sugino T, Malchow J, Schuermann A, Baumeister S, Kwon HB, Maischein HM, Potente M, Herzog W, Stainier DY. Apelin signaling drives vascular endothelial cells toward a pro-angiogenic state. Elife. 2020;9.
185. Wu L, Chen L, Li L. Apelin/APJ system: A novel promising therapy target for pathological angiogenesis. Clin Chim Acta. 2017;466:78-84.
186. Visser YP, Walther FJ, Laghmani el H, Laarse A, Wagenaar GT. Apelin attenuates hyperoxic lung and heart injury in neonatal rats. Am J Respir Crit Care Med. 2010;182(10):1239-50.
187. Kim MS, Park TI, Lee YM, Jo YM, Kim S. Expression of Id-1 and VEGF in non-small cell lung cancer. Int J Clin Exp Pathol. 2013;6(10):2102-11.
188. Ling MT, Lau TC, Zhou C, Chua CW, Kwok WK, Wang Q, Wang X, Wong YC. Overexpression of Id-1 in prostate cancer cells promotes angiogenesis through the activation of vascular endothelial growth factor (VEGF). Carcinogenesis. 2005;26(10):1668-76.
189. Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, Benezra R. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature. 1999;401(6754):670-7.
190. Maw MK, Fujimoto J, Tamaya T. Overexpression of inhibitor of DNA-binding (ID)-1 protein related to angiogenesis in tumor advancement of ovarian cancers. BMC Cancer. 2009;9:430.
191. Cowan CE, Kohler EE, Dugan TA, Mirza MK, Malik AB, Wary KK. Kruppel-like factor-4 transcriptionally regulates VE-cadherin expression and endothelial barrier function. Circ Res. 2010;107(8):959-66.
192. Ghaleb AM, Yang VW. Kruppel-like factor 4 (KLF4): What we currently know. Gene. 2017;611:27-37.

193. Yoshida T, Yamashita M, Horimai C, Hayashi M. Deletion of Kruppel-like factor 4 in endothelial and hematopoietic cells enhances neointimal formation following vascular injury. J Am Heart Assoc. 2014;3(1):e000622.
194. Kim J, Guan KL. mTOR as a central hub of nutrient signalling and cell growth. Nat Cell Biol. 2019;21(1):63-71.
195. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017;169(2):361-71.
196. Niedzwiecka A, Marcotrigiano J, Stepinski J, Jankowska-Anyszka M, Wyslouch-Cieszynska A, Dadlez M, Gingras AC, Mak P, Darzynkiewicz E, Sonenberg N, Burley SK, Stolarski R. Biophysical studies of eIF4E cap-binding protein: recognition of mRNA 5' cap structure and synthetic fragments of eIF4G and 4E-BP1 proteins. J Mol Biol. 2002;319(3):615-35.
197. Rhoads RE, Dinkova TD, Jagus R. Chapter Thirteen - Approaches for Analyzing the Differential Activities and Functions of eIF4E Family Members. In: Lorsch J, editor. Methods in Enzymology. 429: Academic Press; 2007. p. 261-97.
198. Gibbons JJ, Abraham RT, Yu K. Mammalian target of rapamycin: discovery of rapamycin reveals a signaling pathway important for normal and cancer cell growth. Semin Oncol. 2009;36 Suppl 3:S3-S17.
199. Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev. 1999;13(11):1422-37.
200. Gowans GJ, Hawley SA, Ross FA, Hardie DG. AMP is a true physiological regulator of AMP-activated protein kinase by both allosteric activation and enhancing net phosphorylation. Cell Metab. 2013;18(4):556-66.
201. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell. 2008;30(2):214-26.
202. Karar J, Maity A. PI3K/AKT/mTOR Pathway in Angiogenesis. Front Mol Neurosci. 2011;4:51.
203. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, Semenza GL. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res. 2000;60(6):1541-5.
204. Wahdan-Alaswad RS, Song K, Krebs TL, Shola DT, Gomez JA, Matsuyama S, Danielpour D. Insulin-like growth factor I suppresses bone morphogenetic protein signaling in prostate cancer cells by activating mTOR signaling. Cancer Res. 2010;70(22):9106-17.
205. Burgstaller G, Oehrle B, Gerckens M, White ES, Schiller HB, Eickelberg O. The instructive extracellular matrix of the lung: basic composition and alterations in chronic lung disease. Eur Respir J. 2017;50(1).
206. Zhou Y, Horowitz JC, Naba A, Ambalavanan N, Atabai K, Balestrini J, Bitterman PB, Corley RA, Ding BS, Engler AJ, Hansen KC, Hagood JS, Kheradmand F, Lin QS, Neptune E, Niklason L, Ortiz LA, Parks WC, Tschumperlin DJ, White ES, Chapman HA, Thannickal VJ. Extracellular matrix in lung development, homeostasis and disease. Matrix Biol. 2018;73:77-104.
207. Kunz LI, Strebus J, Budulac SE, Lapperre TS, Sterk PJ, Postma DS, Mauad T, Timens W, Hiemstra PS, Group GS. Inhaled steroids modulate extracellular matrix composition in bronchial biopsies of COPD patients: a randomized, controlled trial. PLoS One. 2013;8(5):e63430.
208. Lu Y, Guo H, Sun Y, Pan X, Dong J, Gao D, Chen W, Xu Y, Xu D. Valsartan attenuates pulmonary hypertension via suppression of mitogen activated protein kinase signaling and matrix metalloproteinase expression in rodents. Mol Med Rep. 2017;16(2):1360-8.
209. Mizikova I, Ruiz-Camp J, Steenbock H, Madurga A, Vadasz I, Herold S, Mayer K, Seeger W, Brinckmann J, Morty RE. Collagen and elastin cross-linking is altered during aberrant late lung development associated with hyperoxia. Am J Physiol Lung Cell Mol Physiol. 2015;308(11):L1145-58.
210. Vieira CP, de Oliveira LP, Da Silva MB, Majolli Andre D, Tavares EBG, Pimentel ER, Antunes E. Role of metalloproteinases and TNF-alpha in obesity-associated asthma in mice. Life Sci. 2020;259:118191.
211. Hynes RO, Naba A. Overview of the matrisome--an inventory of extracellular matrix constituents and functions. Cold Spring Harb Perspect Biol. 2012;4(1):a004903.
212. Naba A, Clauser KR, Hoersch S, Liu H, Carr SA, Hynes RO. The matrisome: in silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. Mol Cell Proteomics. 2012;11(4):M111 014647.
213. Hilgendorff A, Parai K, Ertsey R, Navarro E, Jain N, Carandang F, Peterson J, Mokres L, Milla C, Preuss S, Alcazar MA, Khan S, Masumi J, Ferreira-Tojais N, Mujahid S, Starcher B, Rabinovitch M, Bland R. Lung matrix and vascular remodeling in mechanically ventilated elastin haploinsufficient newborn mice. Am J Physiol Lung Cell Mol Physiol. 2015;308(5):L464-78.
214. Pufe T, Harde V, Petersen W, Goldring MB, Tillmann B, Mentlein R. Vascular endothelial growth factor (VEGF) induces matrix metalloproteinase expression in immortalized chondrocytes. J Pathol. 2004;202(3):367-74.
215. Rothhammer T, Braig S, Bosserhoff AK. Bone morphogenetic proteins induce expression of metalloproteinases in melanoma cells and fibroblasts. Eur J Cancer. 2008;44(16):2526-34.
216. Wang X, Khalil RA. Matrix Metalloproteinases, Vascular Remodeling, and Vascular Disease. Adv Pharmacol. 2018;81:241-330.

217. Huang PH, Chen YH, Wang CH, Chen JS, Tsai HY, Lin FY, Lo WY, Wu TC, Sata M, Chen JW, Lin SJ. Matrix metalloproteinase-9 is essential for ischemia-induced neovascularization by modulating bone marrow-derived endothelial progenitor cells. Arterioscler Thromb Vasc Biol. 2009;29(8):1179-84.
218. Mahor D, Kumari V, Vashisht K, Galgalekar R, Samarth RM, Mishra PK, Banerjee N, Dixit R, Saluja R, De S, Pandey KC. Elevated serum matrix metalloprotease (MMP-2) as a candidate biomarker for stable COPD. BMC Pulm Med. 2020;20(1):302.
219. Lepetit H, Eddahibi S, Fadel E, Frisdal E, Munaut C, Noel A, Humbert M, Adnot S, D'Ortho MP, Lafuma C. Smooth muscle cell matrix metalloproteinases in idiopathic pulmonary arterial hypertension. Eur Respir J. 2005;25(5):834-42.
220. Marini JJ. Microvasculature in ventilator-induced lung injury: target or cause? Minerva Anestesiol. 2004;70(4):167-73.
221. Chao CM, El Agha E, Tiozzo C, Minoo P, Bellusci S. A breath of fresh air on the mesenchyme: impact of impaired mesenchymal development on the pathogenesis of bronchopulmonary dysplasia. Front Med (Lausanne). 2015;2:27.
222. Vandamme TF. Rodent models for human diseases. Eur J Pharmacol. 2015;759:84-9.
223. Swanson AM, David AL. Animal models of fetal growth restriction: Considerations for translational medicine. Placenta. 2015;36(6):623-30.
224. Resnick O, Morgane PJ, Hasson R, Miller M. Overt and hidden forms of chronic malnutrition in the rat and their relevance to man. Neurosci Biobehav Rev. 1982;6(1):55-75.
225. Dravet-Gounot P, Morin C, Jacques S, Dumont F, Ely-Marius F, Vaiman D, Jarreau PH, Mehats C, Zana-Taieb E. Lung microRNA deregulation associated with impaired alveolarization in rats after intrauterine growth restriction. PLoS One. 2017;12(12):e0190445.
226. Nawabi J, Vohlen C, Dinger K, Thangaratnarajah C, Klaudt C, Lopez Garcia E, Hirani DV, Karakaya PH, Macheleidt I, Odenthal M, Nusken KD, Dotsch J, Alejandre Alcazar MA. Novel functional role of GH/IGF-I in neonatal lung myofibroblasts and in rat lung growth after intrauterine growth restriction. Am J Physiol Lung Cell Mol Physiol. 2018;315(5):L623-L37.
227. Plank C, Ostreicher I, Hartner A, Marek I, Struwe FG, Amann K, Hilgers KF, Rascher W, Dotsch J. Intrauterine growth retardation aggravates the course of acute mesangioproliferative glomerulonephritis in the rat. Kidney Int. 2006;70(11):1974-82.
228. Chen X, Orriols M, Walther FJ, Laghmani EH, Hoogeboom AM, Hogen-Esch ACB, Hiemstra PS, Folkerts G, Goumans MTH, Ten Dijke P, Morrell NW, Wagenaar GTM. Bone Morphogenetic Protein 9 Protects against Neonatal Hyperoxia-Induced Impairment of Alveolarization and Pulmonary Inflammation. Front Physiol. 2017;8:486.
229. Bai Y, Leng Y, Yin G, Pu X, Huang Z, Liao X, Chen X, Yao Y. Effects of combinations of BMP-2 with FGF-2 and/or VEGF on HUVECs angiogenesis in vitro and CAM angiogenesis in vivo. Cell Tissue Res. 2014;356(1):109-21.
230. Ng YS, Rohan R, Sunday ME, Demello DE, D'Amore PA. Differential expression of VEGF isoforms in mouse during development and in the adult. Dev Dyn. 2001;220(2):112-21.
231. Acarregui MJ, Penisten ST, Goss KL, Ramirez K, Snyder JM. Vascular endothelial growth factor gene expression in human fetal lung in vitro. Am J Respir Cell Mol Biol. 1999;20(1):14-23.
232. Le Cras TD, Markham NE, Tuder RM, Voelkel NF, Abman SH. Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure. Am J Physiol Lung Cell Mol Physiol. 2002;283(3):L555-62.
233. Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, Kang MJ, Cohn L, Kim YK, McDonald DM, Elias JA. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. Nat Med. 2004;10(10):1095-103.
234. Saaristo A, Veikkola T, Enholm B, Hytonen M, Arola J, Pajusola K, Turunen P, Jeltsch M, Karkkainen MJ, Kerjaschki D, Bueler H, Yla-Herttuala S, Alitalo K. Adenoviral VEGF-C overexpression induces blood vessel enlargement, tortuosity, and leakiness but no sprouting angiogenesis in the skin or mucous membranes. FASEB J. 2002;16(9):1041-9.
235. Pulkkinen HH, Kiema M, Lappalainen JP, Toropainen A, Beter M, Tirronen A, Holappa L, Niskanen H, Kaikkonen MU, Yla-Herttuala S, Laakkonen JP. BMP6/TAZ-Hippo signaling modulates angiogenesis and endothelial cell response to VEGF. Angiogenesis. 2020.
236. Rezzola S, Di Somma M, Corsini M, Leali D, Ravelli C, Polli VAB, Grillo E, Presta M, Mitola S. VEGFR2 activation mediates the pro-angiogenic activity of BMP4. Angiogenesis. 2019;22(4):521-33.
237. Alastalo TP, Li M, Perez Vde J, Pham D, Sawada H, Wang JK, Koskenvuo M, Wang L, Freeman BA, Chang HY, Rabinovitch M. Disruption of PPARgamma/beta-catenin-mediated regulation of apelin impairs BMP-induced mouse and human pulmonary arterial EC survival. J Clin Invest. 2011;121(9):3735-46.
238. Joss-Moore L, Carroll T, Yang Y, Fitzhugh M, Metcalfe D, Oman J, Hale M, Dong L, Wang ZM, Yu X, Callaway CW, O'Brien E, McKnight RA, Lane RH, Albertine KH. Intrauterine growth restriction transiently delays alveolar formation and disrupts retinoic acid receptor expression in the lung of female rat pups. Pediatr Res. 2013;73(5):612-20.
239. Thangaratnarajah C, Dinger K, Vohlen C, Klaudt C, Nawabi J, Lopez Garcia E, Kwapiszewska G, Dobner J, Nusken KD, van Koningsbruggen-Rietschel S, von Horsten S, Dotsch J, Alejandre Alcazar MA. Novel role of NPY in neuroimmune interaction and lung growth after intrauterine growth restriction. Am J Physiol Lung Cell Mol Physiol. 2017;313(3):L491-L506.

240. Zana-Taieb E, Pham H, Franco-Montoya ML, Jacques S, Letourneur F, Baud O, Jarreau PH, Vaiman D. Impaired alveolarization and intra-uterine growth restriction in rats: a postnatal genome-wide analysis. J Pathol. 2015;235(3):420-30.
241. Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM. Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1, and TIE-2 in human infants dying with bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001;164(10 Pt 1):1971-80.
242. Klinger G, Sokolover N, Boyko V, Sirota L, Lerner-Geva L, Reichman B, Israel Neonatal N. Perinatal risk factors for bronchopulmonary dysplasia in a national cohort of very-low-birthweight infants. Am J Obstet Gynecol. 2013;208(2):115 e1-9.
243. DeLisser HM, Helmke BP, Cao G, Egan PM, Taichman D, Fehrenbach M, Zaman A, Cui Z, Mohan GS, Baldwin HS, Davies PF, Savani RC. Loss of PECAM-1 function impairs alveolarization. J Biol Chem. 2006;281(13):8724-31.
244. Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C, Huyck H. Angiogenic factors and alveolar vasculature: development and alterations by injury in very premature baboons. Am J Physiol Lung Cell Mol Physiol. 2002;282(4):L811-23.
245. Herwig MC, Muller KM, Muller AM. Endothelial VE-cadherin expression in human lungs. Pathol Res Pract. 2008;204(10):725-30.
246. Vestweber D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. Arterioscler Thromb Vasc Biol. 2008;28(2):223-32.
247. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM. Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol. 2000;156(4):1363-80.
248. Han W, Guo C, Liu Q, Yu B, Liu Z, Yang J, Deng C. Aberrant elastin remodeling in the lungs of O(2)-exposed newborn mice; primarily results from perturbed interaction between integrins and elastin. Cell Tissue Res. 2015;359(2):589-603.
249. Kumarasamy A, Schmitt I, Nave AH, Reiss I, van der Horst I, Dony E, Roberts JD, Jr., de Krijger RR, Tibboel D, Seeger W, Schermuly RT, Eickelberg O, Morty RE. Lysyl oxidase activity is dysregulated during impaired alveolarization of mouse and human lungs. Am J Respir Crit Care Med. 2009;180(12):1239-52.
250. Jiao Y, Li G, Li Q, Ali R, Qin L, Li W, Qyang Y, Greif DM, Geirsson A, Humphrey JD, Tellides G. mTOR (Mechanistic Target of Rapamycin) Inhibition Decreases Mechanosignaling, Collagen Accumulation, and Stiffening of the Thoracic Aorta in Elastin-Deficient Mice. Arterioscler Thromb Vasc Biol. 2017;37(9):1657-66.
251. Ben-Yosef Y, Miller A, Shapiro S, Lahat N. Hypoxia of endothelial cells leads to MMP-2-dependent survival and death. Am J Physiol Cell Physiol. 2005;289(5):C1321-31.
252. Cheng XW, Kuzuya M, Nakamura K, Maeda K, Tsuzuki M, Kim W, Sasaki T, Liu Z, Inoue N, Kondo T, Jin H, Numaguchi Y, Okumura K, Yokota M, Iguchi A, Murohara T. Mechanisms underlying the impairment of ischemia-induced neovascularization in matrix metalloproteinase 2-deficient mice. Circ Res. 2007;100(6):904-13.
253. Huang S, Van Arsdall M, Tedjarati S, McCarty M, Wu W, Langley R, Fidler IJ. Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. J Natl Cancer Inst. 2002;94(15):1134-42.
254. Ye CL, Rabinovitch M. Inhibition of elastolysis by SC-37698 reduces development and progression of monocrotaline pulmonary hypertension. Am J Physiol. 1991;261(4 Pt 2):H1255-67.
255. Vieillard-Baron A, Frisdal E, Raffestin B, Baker AH, Eddahibi S, Adnot S, D'Ortho MP. Inhibition of matrix metalloproteinases by lung TIMP-1 gene transfer limits monocrotaline-induced pulmonary vascular remodeling in rats. Hum Gene Ther. 2003;14(9):861-9.
256. Khazaee R, McCaig LA, Yamashita C, Hardy DB, Veldhuizen RAW. Maternal protein restriction during perinatal life affects lung mechanics and the surfactant system during early postnatal life in female rats. PLoS One. 2019;14(4):e0215611.

Erklärung zur Dissertation

gemäß der Promotionsordnung vom 12. März 2020

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Teilpublikationen:

Kuiper-Makris C, Zanetti D, Vohlen C, Fahle L, Muller M, Odenthal M, Felderhoff-Muser U, Dotsch J, Alejandre Alcazar MA. Mendelian randomization and experimental IUGR reveal the adverse effect of low birth weight on lung structure and function. Sci Rep. 2020;10(1):22395.

Datum, Name und Unterschrift

Declaration for the doctoral thesis (dissertation)

according to the doctoral regulations published 12th March 2020

"I hereby declare that I have completed the present dissertation independently and without the use of any aids or literature other than those referred to. All passages that have been taken, either literally or in sense, from published and unpublished works, are marked as such. I declare that this dissertation has not been submitted to any other faculty or university; that - apart from the partial publications and included articles and manuscripts listed below - it has not yet been published, and that I will not publish the dissertation before completing my doctorate without the permission of the PhD Committee. I am aware of the terms of the doctoral regulations. In addition, I hereby declare that I am aware of the “Regulations for Safeguarding Good Scientific Practice and Dealing with Scientific Misconduct” of the University of Cologne, and that I have observed them during the work on the thesis project and the written doctoral thesis. I hereby commit myself to observe and implement the guidelines mentioned there in all scientific activities. I assure that the submitted electronic version is identical to the submitted printed version".

Partial publications of the thesis:

Kuiper-Makris C, Zanetti D, Vohlen C, Fahle L, Muller M, Odenthal M, Felderhoff-Muser U, Dotsch J, Alejandre Alcazar MA. Mendelian randomization and experimental IUGR reveal the adverse effect of low birth weight on lung structure and function. Sci Rep. 2020;10(1):22395.

Date, name, and signature

Erklärung zur Masterarbeit

Im Jahr 2016 habe ich an der Universität Maastricht meine Masterarbeit mit dem Titel: '*The influence of Intra-Uterine Growth Restriction on Pulmonary Angiogenesis in a Rat Model´* vorgelegt, das Vorarbeiten für das originalen Paper (Figure 1B, 1C, 2C, 3A, 3B (P3 und P23) und 4A) enthielt. Für die Masterarbeit habe ich mich überwiegend auf das Effekt von IUGR und Aufholwachstum auf die Endothelzellfunktion fokussiert, mittels qPCR und Immunoblotting der VEGF- und BMP-Signalwegen. Für die vorliegende Doktorarbeit habe ich mich zugelegt auf den Einfluss von IUGR auf *nutrient sensing* und die histologischen Folgen von IUGR auf die Lunge hinsichtlich der Gefäßentwicklung und Gewebe Umbau. Dazu habe ich histologische Färbungen für Endothelzelmarkern (in der Zusammenarbeit mit Frau M. Odenthal und Frau M Mueller), Elastin und Kollagen (nicht Teil der Veröffentlichung) durchgeführt und kwantifiziert, als auch die mRNA Expression von Elastin und Kollagen untersucht mittels qPCR. Ich habe Immunoblots für 4E-BP1 und AMPKα durchgeführt und analysiert, zur Beurteilung der zentrale *nutrient sensing* Signalkaskaden. Außerdem habe ich in der Zusammenarbeit mit Frau Vohlen die Protease (MMP2 und MMP9) Aktivität im Lung Gewebe untersucht. Letztens, ist ein wichtiger Teil der Veröffentlichung die Daten der Mendelian Randomisation Studie, die durch geführt wurde von der Arbeitsgruppe von Frau D. Zanetti in Stanford. Ich war aktiv beteiligt an den Entwurf, als auch die inhaltliche Diskussion der Ergebnisse. Die hier vorliegende kumulative Arbeit, inklusive den veröffentlichten Paper, enthält entsprechend neue Daten, als auch eine komplett unabhängig von der Vorarbeit geschriebene Einleitung (*Introduction*) und Diskussion (*Discussion*). Somit erkläre ich, dass den vorliegenden Dissertationsschrift nicht im Inland noch im Ausland in gleicher Form einer anderen Prüfungsbehörde vorgelegt wurde.

Gedruckt von Druckerei **Hundt Druck GmbH**, Köln