

Characterization of vascular remodeling associated with pulmonary hypertension in miR-200b knockout mice

By

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Abstract

Introduction: Pulmonary hypertension is one of the main causes of death in congenital diaphragmatic hernia. It results from thickening of the medial and adventitial layers in the lung vessels leading to increased vessel resistance and ultimately heart failure. The underlying pathogenesis of pulmonary hypertension is poorly understood. We hypothesized that microRNA-200b (miR-200b) plays a role in vascular remodeling associated with pulmonary hypertension. To better understand the role of miR-200b we created miR-200b knockout (KO) mice and aimed to 1) evaluate the morphological pulmonary vasculature changes in miR-200b KO mice and 2) to determine the role of miR-200b in pulmonary hypertension by targeting of the vascular endothelial growth factor (VEGF) signaling pathway.

Method: Verhoeff-van Gieson (VVG) staining was used to measure the medial, arterial and adventitial wall thickness of the lung vessels. VEGF-A and its receptors VEGFR-1 and VEGFR-2 expression were assessed using immunohistochemistry (IHC). Micro-computed tomography (micro-CT) was optimized and applied to demonstrate the complexity of the pulmonary vasculature at the microlevel (8 μ m) with high resolution, quantitative, three -dimensional images.

Results: Vascular remodeling assessment showed that miR-200b KO lungs have 35% increased arterial wall thickness, 47% medial wall thickness and 32% adventitial wall thickness in pulmonary vessels compared to normal lung ($P < 0.0001$). The most significant structural changes were observed in arterioles with an external diameter less than 20 or 40 μ m in the miR-

200b KO group. IHC results showed that VEGF-A was downregulated and VEGFR-1 was upregulated in miR-200b KO lungs. However, VEGFR-2 expression did not show any difference between miR-200b KO and WT mice. Micro-CT data did not show any difference between miR-200b KO and WT mice.

Conclusion: Pulmonary hypertension in miR-200b KO mice is associated with changes in vascular morphology. Our results suggest that the absence of miR-200b results in pulmonary hypertension by vascular remodeling of the pulmonary vessels especially in arterioles. MiR-200b could also contribute to pulmonary hypertension by downregulation of VEGF-A and upregulation of VEGFR-1.

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Dedications

*I would like to dedicate my thesis
to my adorable parents, Mohammadhasan and Zahra,*

For your endless love, support and care.

Without you, none of my success would be possible.

Thanks for making my dreams true!

*To my dearest sisters, Sepideh and Sahar,
for your inspiration and encouragement
that makes me believe I can be the best I can be.*

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List of Abbreviations

3D	three -dimensional
AD	adventitial diameter
ANGPT	Angiopoietin
BMP	bone morphogenetic protein
BNP	B-type natriuretic peptide
CDH	congenital diaphragmatic hernia
cGMP	cyclic guanosine monophosphate
COPD	chronic obstructive pulmonary disease
E	embryonic day
ECMO	extracorporeal membrane oxygenation
ED	external diameter
EMT	epithelial-to-mesenchymal transition
ET	endothelin
FETO	fetoscopic endoluminal tracheal occlusion
FGF	fibroblast growth factor
ID	internal diameter
IHC	immunohistochemistry
IVC	inferior vena cava
KO	knockout
Micro-CT	micro-computed tomography
miR-200b	MicroRNA-200b
NO	Nitric oxide
NOS	nitric oxide synthase
NT-proBNP	N-terminal proBNP
O/E LHR	observed over expected lung-to-head ratio

PAH	pulmonary arterial hypertension
PAT	pulmonary acceleration time
PBS	phosphate buffered saline
PDGF-B	platelet-derived growth factor B
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PVR	pulmonary vascular resistance
RISC	RNA-induced silencing complex
TGF- β	transforming growth factor beta
VEGF	vascular endothelial growth factor
VVG	Verhoeff Van Gieson
WT	wildtype

CHAPTER 1. INTRODUCTION

1.1 Overview of congenital diaphragmatic hernia (CDH)

Congenital diaphragmatic hernia (CDH) is a developmental defect of the diaphragm in newborns, resulting in herniation of abdominal organs like intestine or stomach into the thoracic cavity and undeveloped lung (Figure 1) [1]. CDH accounts for approximately 8% of the important congenital abnormalities [2] with a prevalence of 1 in 2000-3000 newborns [3]. It means every 10 minutes one baby is born with CDH worldwide. The hole in the diaphragm can be repaired after birth by surgery, but mortality and morbidity of CDH are associated with pulmonary hypoplasia, pulmonary hypertension and cardiac dysfunction [4] [5].

Hypoplastic lungs have thickened mesenchymal cells, decreased lung volume, deficient surfactant, and fewer airway branches [6]. Pulmonary hypoplasia occurs owing to two developmental insults. The first insult appears before diaphragm development and affects both lungs. The second one occurs after closing of the diaphragm only in the ipsilateral lung due to mechanical compression of the lung [7]. Pulmonary hypertension associated with CDH is identified by increased thickness of the medium to large vessels and muscularization of small arterioles [8]. These changes increase the vascular stiffness which contributes to right ventricular afterload. As a result, ventricular hypertrophy and heart defect contribute to the mortality in CDH patients [9].

Despite successful improvement in the care of CDH patients, the morbidity rate remains nearly 100% [2] with 30 to 50% mortality [10]. A better understanding of the molecular mechanisms underlying CDH and development of neonatal care could improve the survival rate of CDH [11]. Over the past years, advanced diagnostic techniques provide an opportunity to predict high risk

pregnancies with minimally invasive procedures on the fetus. To predict the degree of pulmonary hypoplasia in CDH lungs, ultrasound can be used to estimate observed/expected lung-to-head ratio (O/E LHR) between 22 to 32 weeks of gestational age. Fetuses at 20-26 weeks of gestation with an O/E LHR < 0.6 cannot survive however, babies with an O/E LHR > 1.35 have a 100% chance of survival [1] [6]. A recent study has shown a correlation between O/E LHR and pulmonary hypertension due to CDH and the result suggested that newborns with an O/E LHR $\leq 45\%$ have a higher risk of increased right ventricular systolic pressure. While postnatal treatment improves the pulmonary hypertension, pulmonary hypoplasia is not fixed and shows up as an irreversible defect in CDH infants [12]. Fetal magnetic resonance imaging is an imaging tool which has recently been used to assess liver herniation and total lung volume in moderate and severe cases [13] [14].

The etiology of CDH remains unknown in ~85% of patients and is thought to be multifactorial. CDH can be associated with congenital anomalies like gastrointestinal, cardiovascular and musculoskeletal or with chromosomal abnormalities, genetic syndromes and environmental factors [15]. Additionally, several studies revealed that maternal exposure to alcohol, cigarette smoking, and antimicrobial drugs could increase the risk of CDH [16] [17]. Recent studies suggest that epigenetic factors such as microRNAs [18] or somatic mutations can contribute to the development of CDH [1].

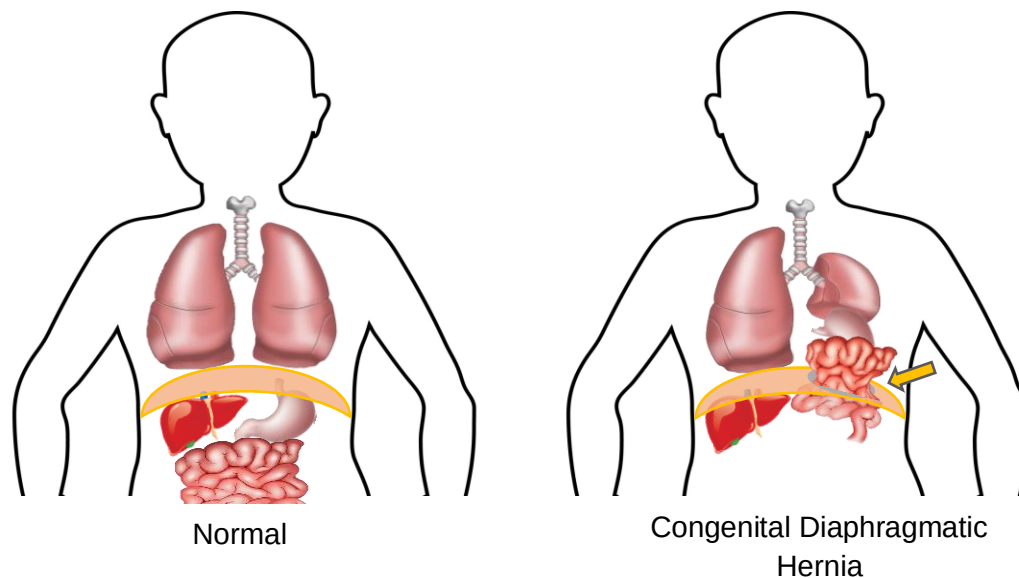


Figure 1. Schematic view of congenital diaphragmatic hernia. In CDH babies (left image) the diaphragm does not form properly allowing herniation of abdominal organs into the thoracic cavity and abnormal lung development.

1.2 Normal pulmonary vascular development during lung development

Formation of the vascular network has two different phases: 1) proliferation and differentiation of the primitive vasculature 2) maturation and integration of pulmonary vessels by counteraction and remodeling, which results in maintenance of pulmonary circulation function [19]. Pulmonary vascular development has a very close relation with the growth of alveoli and airways in a mechanism regulated by the interaction between endothelial and epithelial cells development. Thus, any disruption in one system can have harmful effects on the function of the other one. As most of the described experiments were done in animal models, time points of different lung development stages are shown for both human and mice.

Lung development can be divided into five morphological stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar. It starts with organogenesis at an embryonic stage of 3 to 5 weeks of gestation in humans and Embryonic day (E)9.5 to E14.2 in mice. First, the lung derives from foregut endoderm then, the interactions between the ventral side of the foregut endoderm and the surrounding mesenchymal tissue leads to the development of the main bronchi. Simultaneously, the pulmonary trunk is originated from the truncus arteriosus and attaches to the pulmonary arteries branch which themselves originate from the sixth pair of the aortic arches. Pulmonary artery branches nourish the lung buds and ultimately form the main pulmonary arteries [20] [21]. At the same time, pulmonary veins originate from the splanchnic mesoderm surrounding the foregut endoderm [22].

Subsequently, in the pseudoglandular stage (human ~5–17 weeks, mouse E11–E17) the bronchial tree undergoes constant dichotomous branching followed by planar and orthogonal bifurcation branching to form the conducting airways and blind-ending terminal bronchioles [23]. At the beginning of this stage, the epithelial cells of the proximal airways are undifferentiated however, throughout this process; they differentiate into goblet, secretory and ciliated cells. The sixth aortic arch grows to the left and right proximal pulmonary vessels parallel to development of the airways, establishing the broncho-arterial bundle. During the next sixth weeks in the canalicular stage, the central artery and vein expand the network of the peripheral capillaries. Concurrently, the last generations of bronchioles and the acinar region develop (human ~16–25 weeks, mouse E18–19). Furthermore, the epithelial cells differentiate into type I and II pneumocytes and alveolar type II pneumocytes start to produce and release surfactant [23]-[26]. The saccular stage starts at week 24 of gestation to week 38 in human and E20 to term in mouse. During this stage, the number of type I pneumocytes increases to cover

approximately 95% of the alveolar sac. Also, tremendous capillary growth forms an air-blood barrier which later allows for gas exchange and plays an important role to provide O₂ for organs and eliminate excessive CO₂ [27] [28]. In the alveolar stage, (human 36 weeks in utero to approximately 7 years after birth, mouse from birth to ~7 weeks after birth) a pivotal transition from prenatal life to postnatal life occurs which ultimately leads to normal breathing in newborns. Alveolarization and angiogenesis form massive alveolar ducts and a network of micro-vessels, as a functional surface, with an estimated size of approximately 70 m² in adult human for gas exchange [29].

1.3 Molecular regulation of pulmonary vascular development

Vascular development depends on the normal function of two major processes: vasculogenesis and angiogenesis. Vasculogenesis occurs when new blood vessels generate from the differentiation of mesodermal progenitor cells into mature endothelial cells. Angiogenesis is the process when capillary buds growth from pre-existing blood vessels. Regulation of the pulmonary circulation is a complex spatio-temporal process related to various cell signaling and transcription factors that are not properly understood to date [30]. Talking about all components and mediators is beyond the scope of my thesis. Here, I mainly discuss the most important signaling pathways involved in vascular development including vascular endothelial growth factor (VEGF), Platelet-derived growth factor B (PDGF-B), Angiopoietin (ANGPT) and transforming growth factor beta (TGF- β) [31]. From different regulators, the VEGF signaling pathway appears to be one of the most critical angiogenic signaling factors for a well-developed

pulmonary circulation [32]. The role and importance of VEGF will be explained in detail later in the section “pathways involved in pulmonary hypertension associated with CDH”.

PDGF-B promotes the endothelial cells growth in the newly formed tube to recruit pericytes and establish vessel wall integrity and maintenance. PDGF-B is secreted by the endothelium of arteries and immature capillaries. This molecule signals through two tyrosine kinase cell-surface receptors, PDGFR α and PDGFR β . These receptors interact with the SH-2 domain-containing proteins and target various cells like smooth muscle cells, pericytes and fibroblasts [33]. Mutation of *Pdgfb* and *Pdgfrb* together lead to a reduction in the recruitment and development of perivascular cells however, mural cells primarily develop around vessels [34]. Co stimulation of VEGF-A and fibroblast growth factor (FGF-2) improve angiogenesis by increasing the expression of PDGF-B and PDGFR β [35].

ANGPT signaling has a critical role in the integrity of the vessels and vascular remodeling. It binds to Tie receptors, Tie 1 and Tie 2. These receptors are expressed from the earliest stage of embryonic development to adult life. Binding angiopoietin to Tie 2 activates downstream pathways involved in endothelial cell to cell interaction, vessel branching, and cell survival/apoptosis. Mutation of Tie 2 results in embryonic lethality in midgestation due to vessel malformation and instability. Interestingly, over-expression of Tie 2 causes a vascular remodeling phenotype similar to the loss of expression of Tie 2. The role of the Tie 1 receptor was discovered recently. Tie 1 can increase the agonistic activity of angiopoietin through negative feedback and decreases Tie 2 activity [31] [32] [36].

Bone morphogenetic protein (BMP) family is a member of the TGF- β superfamily with a crucial role in lung development, angiogenesis and organogenesis in humans and mice. BMP signaling is controlled by the interaction of serine/threonine kinase receptors and ligand, resulting in

phosphorylation of Smad 1, 5 and 9. These Smad proteins are the main transcription factors mediating downstream of BMP receptors. Phosphorylation of Smad 1/5/9 leads to a complex with Smad 4, which translocates to the nucleus and interacts with other coreceptors and coactivators to modulate target gene transcription in a tissue/cell-specific manner [37]. Different members of the BMP family have a pro or anti-angiogenic activity. BMP2 stimulates tip cell generation via delta-like ligand 4 and kinase insert domain receptor while BMP6 promotes stalk cell generation through Smad 1/5 [31].

1.4 Pathways involved in pulmonary hypertension associated with CDH

Molecular pathogenesis of pulmonary hypertension in CDH is multifactorial, associated with different genes and proteins. Among several involved signaling pathways, the retinoic acid pathway, the endothelin (ET) pathway, the nitric oxide (NO) and the VEGF pathway have been the focus of attention in both human CDH and CDH animal models. However, only a few studies addressed the reason for the pathologic pulmonary vasculature in CDH patients and it remains under-investigated [38]. A brief description of each pathway with a focus on the VEGF signaling pathway is provided below.

1.4.1 Retinoic acid signaling pathway

Retinoic acid is required in embryogenesis and particularly early lung development. It is metabolized to vitamin A (retinol). Pulmonary vascular defects and abnormal diaphragmatic growth in CDH may result from changes in the retinoic acid signaling pathway. Nitrofen, a teratogenic herbicide, is widely used to study CDH in a rodent animal model. This chemical

inhibits the activity of retinal dehydrogenase and administration of nitrofen to pregnant rodents at 9 days of gestation (E9) induces diaphragmatic defects in 50 to 60 % of the offspring and pulmonary hypoplasia in 100% of pups [39]. Also, the offspring demonstrates pulmonary hypertension with increased arterial wall thickness, decreased vascular density in the lung and inappropriate responses to vasodilator treatment [40] [41].

Neo-muscularization and reduced airway branching in CDH may be the result of disturbances in the RA- β -FGF10 interactions. The lower levels of retinoic acid during embryonic development results in overexpression of TGF- β . TGF- β is a member of the transforming growth factor beta superfamily including various growth factors with a critical role in the stimulation of pulmonary vascular growth and airway branching. Abnormal expression of TGF- β downregulates the expression of the growth factor FGF-10, resulting in disturbed budding and increased immature differentiated perivascular cells from mesenchymal cells and therefore lung hypoplasia [37]-[39].

1.4.2 Endothelin signaling pathway

Endothelin peptides include 3 isoforms: endothelin 1 (ET-1), endothelin 2 (ET-2) and endothelin 3 (ET-3), of which ET-1 is the most common recognized endothelin [45]. ET-1 is mainly identified within the lung and produced by endothelial cells of the pulmonary artery. It has a pivotal role in pulmonary vascular homeostasis by keeping the balance between vasoconstrictor/vasodilator factors [41] [42]. ET-1 induces its effects by activating two different G-protein receptors: ET-A and ET-B. When ET-1 binds to ET-A, which is present in the cell surface of vascular smooth muscle cells, it promotes vasoconstriction by stimulating the release

of cytosolic calcium and cell proliferation by activating phospholipase C. In contrast, ET-B is mostly located in the pulmonary arterial smooth muscle cells and cell membrane of EC. It induces vasodilation by stimulating the release of prostacyclin and NO [43] [44] [46]. The elevated level of ET-1 expression has been found in all types of pulmonary hypertension, including pulmonary hypertension related to CDH [47]-[51]. Increased expression of ET-A and ET-B receptors have been shown in both human infants with CDH [52] and nitrofen-induced CDH in the stages of embryonic development (E15) [53] [54] [55]. Higher concentration of ET-1 and ET-A are associated with more vascular constriction and increased pulmonary blood pressure which results in the severe disease and poor outcome [15].

1.4.3 Nitric oxide signaling pathway

NO is involved in several physiological/pathophysiological vital processes such as angiogenesis or lung development in mammals. NO is synthesized by nitric oxide synthase (NOS) and diffused from endothelial cells to the smooth muscle cells to induce vasodilation in pulmonary vessels through the activation of cyclic guanosine monophosphate (cGMP) [56]. NOS expression normally increases during late gestation and reaches a peak in the near term to facilitate the transition from fetal to neonatal life [57]. Mechanical factors such as breathing movements, ventilation, and shear stress can stimulate the production of NO at birth. Studies on NOS expression in the human CDH lung and nitrofen-exposed rat model have yielded various results. While decreased expression of NOS and NO production has been observed in early stages (saccular) in nitrofen-exposed CDH pups [58] [59] [60], high levels of NOS expression have been shown in late gestation (E21) of the CDH lung [55] [61] [62]. Variable results for the

expression of NO between the studies could be explained by using different methods to harvest lungs from the fetus. In the studies with higher expression of NOS, first, fetuses were delivered by cesarean section then euthanized [50] [56] [57], however in the studies with lower expression of NOS, pups did not have the chance to breath because dams were euthanized then whole lung tissues from sacrificed fetuses harvested [58] [59] [60]. In human studies, NOS expression has been found to be decreased at birth in newborns with CDH [63], [64], whereas a few days after birth or extracorporeal membrane oxygenation (ECMO) therapy level of NOS starts to increase [65] [66].

1.4.4 VEGF signaling pathway

The VEGF family members, VEGF-A, -B, -C, -D and placental growth factor are considered as a part of the PDGF family. VEGF-A is a pluripotent growth and permeability factor and has a critical role in the stimulation of angiogenesis/vasculogenesis during adult life and embryonic development [67] [68]. VEGF not only has an important role in normal biological processes but also, plays a role in several pathological conditions such as tumor angiogenesis [69], pulmonary hypertension and intraocular neovascular disorders [70].

In mid-gestation, VEGF-A mRNA is expressed in epithelium and mesenchymal cells, but in late gestation and postnatally it is only expressed in epithelial cells to promote branching morphogenesis. Ferrara *et al.* demonstrated deletion of only a single VEGF-A allele can cause embryonic mortality in mice at an early stage (E11) from severe disorganization of the vascular bed and defective formation of angiogenic sprouts [71]. Alternative splicing of the *Vegfa* gene leads to the formation of four mRNA species, encoding four isoforms: VEGF-A₁₂₀, -144, -164, and -

¹⁸⁸ in rat and mouse. Isoforms have different affinity for heparin sulfate proteoglycans and VEGF receptors, suggesting a distinct function for them during vascular development [72]. Furthermore, the pattern of isoforms expression varies during organ development and adulthood. As an example, a lower level of VEGF-A₁₈₈ mRNA is identified in the early stage (E13) however, the expression increased during the late stages, canalicular and saccular. In adult mice, VEGF-A₁₈₈ is widely expressed in type II pneumocytes cells in the lung [73].

VEGF-A increased the mitosis and migration of endothelial cells through interaction with two high-affinity receptors, VEGFR-1 or Flt-1 and VEGFR-2 or Flk-1 [74]. Both VEGFR-1 and VEGFR-2 have been identified in vascular endothelial cells and epithelial cells [75]. VEGFR-2 is upregulated in gestation, mediates vascular permeability and development by promoting vascular endothelial cells formation, division and migration. VEGFR-1 has a higher affinity than VEGFR-2 to bind to VEGF-A and negatively regulates VEGF-A signaling by preventing VEGFR-2 activity [76]. VEGFR-1 increases in the late stages of embryogenesis (E21), limiting the production of endothelial cells to improve the maintenance of the vessel wall, maturation of endothelial cells and stabilization of vessels [77]. Under hypoxic conditions, epithelium and the medial layer of pulmonary artery produce hypoxia inducible factor, a transcription factor with oxygen-sensitive subunits, stimulating the release of VEGF-A. Then, the binding of VEGF-A to tyrosine kinase receptors leads to dimerization and activation of receptors. This could be a trigger for downstream intracellular signaling cascades resulting in cell proliferation, morphogenesis and, survival [78].

In the nitrofen CDH model, studies of the VEGF-A expression have shown conflicting results. Both increased and decreased VEGF-A level have been reported in the same model [74]. In contrast, changes in the level of VEGF-A receptors (VEGFR-1 and -2) have produced consistent

results with downregulation in the late lung development stage from E18 to E21 in the nitrofen pups [79] [80]. Human studies have shown decreased VEGF-A levels in lungs of CDH fetuses. Interestingly, no significant difference has been reported in the expression of VEGF-A between CDH infants and healthy babies at birth. However, the levels of VEGF-A expression increased in the pulmonary arteries, particularly the small ones ($>200\text{ }\mu\text{m}$), a few days after birth. This could be explained as a compensation mechanism by the vascular bed to decrease the negative effects of pulmonary hypertension [81].

1.5 Vascular structural changes in pulmonary hypertension

The vascular wall of an artery includes three layers: the tunica intima, the tunica media and the tunica adventitia with specific cells in each layer. The tunica intima contains internal elastic lamina and endothelial cells surrounded by vascular smooth muscle cells in the media layer. The contribution of the intima to vascular remodeling is not clear. In normal conditions, 10% of the overall vessel thickness allocates to the intima. However, in severe pulmonary hypertension this number increases to 25% [51]. The media layer is mainly composed of vascular smooth muscle cells. In pulmonary hypertension, media layer has a critical role in vascular remodeling by regulation of vascular tone and muscularization. Vascular smooth muscle cells display heterogenicity and diverse phenotypes based on their function. They can be distinguished by shape, size, expression of cytoskeletal or contractile markers and response to growth factors/inhibitors [82]. The media layer normally represents approximately 20% of the fractional thickness, whereas in PAH the media thickness is almost 60% and media remodeling mostly occurs during chronic hypoxia. The adventitial layer is comprised of collagen fibers, external

elastic lamina, and inflammatory cells. Adventitia remodeling occurs in many vascular disorders. Accurate tracing of the adventitia boundaries is challenging in morphological assessment and therefore introduces significant pitfalls for data analysis [51].

1.6 Pulmonary hypertension of newborns and adult

Pulmonary hypertension is defined by a pulmonary arterial systolic pressure more than 35 mmHg, mean pulmonary arterial pressure more than 25 mmHg at rest and 30 mmHg after exercise. Pulmonary hypertension in newborns, infants, and young adults is a complex condition related to various diseases with similar characteristics versus different underlying etiologies. Pediatric pulmonary hypertension can appear as primary due to congenital heart disease, genetic syndromes and persistent pulmonary hypertension of the newborn or it can be associated with other disorders such as respiratory disease (alveolar capillary dysplasia, bronchopulmonary dysplasia), liver disease, [83] and CDH [84]. Adult pulmonary hypertension occurs initially with a drug-induced, genetic defect and idiopathic pulmonary arterial hypertension. Whereas, sleep-disordered breathing, alveolar hypoxia, chronic obstructive pulmonary disease (COPD) are categorized as secondary causes of pulmonary hypertension in adults.

The World Health Organization classifies pulmonary hypertension into five groups according to the causes and pathophysiological characteristics: pulmonary arterial hypertension (PAH) (group 1), pulmonary hypertension with left heart disease (group 2), pulmonary hypertension due to lung diseases and/or hypoxia (group 3), chronic thromboembolic pulmonary hypertension (group 4) and pulmonary hypertension with unclear and/or multifactorial mechanisms (group 5) [80] [87]. Pulmonary hypertension of the newborn and pulmonary hypertension in the young adult are

subgroups of PAH (group 1). PAH is a chronic progressive disease with increased pulmonary vascular resistance (PVR) leading to right heart failure and ultimately death if untreated [85] [86]. Most of the histopathological changes in adults and infant's pulmonary hypertension are similar and both groups have endothelial and vascular abnormalities. However, infants have a thicker medial area in arteries. In pulmonary hypertension of the newborn precursor cells like pericytes differentiate into smooth muscle cells, although studies on hypoxia-induced PAH showed that circulating progenitor cells could contribute to muscularization of the vessels in adult pulmonary hypertension as well [87]. The diagnostic protocol for both populations is the same with elevated PVR and pulmonary blood pressure, while infants present less with heart failure and more with syncope compared to adults [88]. Recently, biomarkers have been used for diagnostic purposes in numerous fields like cancer or cardiovascular diseases. B-type natriuretic peptide (BNP) and its N-terminal proBNP (NT-proBNP) are produced by cardiac cells in response to stretch due to excessive pressure/volume to heart ventricles. Interestingly, both serum BNP and NT-proBNP are greatly increased in adult pulmonary hypertension, not pulmonary hypertension of the newborns. Assessment of such biomarkers indicates the severity and progression of pulmonary hypertension [89].

1.6.1 Adaptation at birth

A transition from prenatal pulmonary circulation to normal postnatal blood circulation is a critical process including various structural and mechanical changes in pulmonary endothelial and smooth muscle cells which could decrease PVR. Before birth, the placenta is considered as a primary organ for gas exchange and the PVR is high due to contraction of the umbilical and

pulmonary arteries. Also, the foramen ovale and ductus arteriosus shunts facilitate the direct connection between the cardiac blood flow and the systemic circulation. After birth, oxygenation transfers from the placenta to the lungs as a result of breathing, shear stress, mechanical ventilation and pulmonary inflation in dilated vessels. Moreover, with the oxygen levels increasing, the pulmonary blood flow increases noticeably, fetal shunts close within a few minutes after birth and pulmonary blood pressure decreases. The PVR shows a decrease within seconds to hours after birth and then it declines gradually. During normal circulatory transition, the decrease in the PVR can be observed in three different stages. In the early stage, a drastic decline in the PVR occurs within minutes after birth by the mechanical expansion of the lungs and the reduced surface tension in the alveoli. In mid-stage, PVR drops in minutes to hours after birth because of the production in endothelial cells of vasodilating factors such as bradykinin, nitric oxide and prostacyclin. The final stage of PVR reduction proceeds gradually for days to weeks after birth. This stage initiates with changing in the shape of smooth muscle cells associated with the reorganization of the actin cytoskeleton and contractile filaments. Vessel's architectural remodeling occurs along with a decrease in the PVR. Large and small arteries have round smooth muscle cells and a thickened wall with a narrow lumen in utero [90]. However, they obtain a spindle shape and the arteries obtain a wider lumen following circulatory adaptation. This vital process is different in newborns with pulmonary hypertension. Elastic fibers in the adventitial layer do not show changes in normal babies before or after birth, whereas, in babies with pulmonary hypertension, the adventitial area has an outward expansion in large elastic pulmonary arteries as a result of severe hypertrophy in the adventitial layer and mild hypertrophy in the medial layer. In arterioles, most of the remodeling changes are observed

in the medial layer with inward muscularization resulting in a narrower lumen and an increase in the media-to-lumen ratio [91] [44].

1.7 Pulmonary hypertension associated with CDH

Based on the updated clinical classification of pulmonary hypertension, some pathophysiological characteristics of pulmonary hypertension due to CDH are like that of pulmonary hypertension of the newborns (subgroup1). However, it has some characteristics like pulmonary hypertension due to lung disease (subgroup 3). Therefore, it is not easy to classify pulmonary hypertension related to CDH in a specific group. Pulmonary hypertension associated with CDH starts by abnormal vascular development before birth, followed by functional and structural changes in the pulmonary vessels postnatally. This results in a cardiopulmonary disorder and impairs oxygenation and ventilation. Vascular structural changes in pulmonary hypertension related to CDH can be identified by decreased angiogenesis, increased media and adventitia thickness of arteries, changes in molecular pathways and inappropriate responses to vasoactive mediators [82] [83]. Studies in the hypoxia-induced rat model revealed that vascular remodeling in pulmonary hypertension are entirely reversible after recovery in normoxia. However, changes in the heart structure such as right ventricular weigh and right ventricular systolic pressure only partially recovered [84].

1.8 Management and treatment of pulmonary hypertension in CDH

Recently, the survival rate of CDH has increased thanks to improvement in treatment strategies. However, the management of pulmonary hypertension is still complicated due to the diversity of

the underlying etiology. Fetoscopic endoluminal tracheal occlusion (FETO) is used antenatally to accelerate lung development in CDH patients. In this approach, a balloon is inserted into the trachea for 4 to 6 weeks to occlude the airways and increase the growth and size of air sacs by preventing lung fluid from escaping the airways [92]. In a recent study, authors reported that FETO in mild to moderate CDH patients could resolve pulmonary hypertension and in severe cases reduce it by one year after birth [93]. Despite the advantages of FETO, associated risks like damage to the airways, fetal blood loss, and pre-term labor should be considered.

The postnatal management of CDH includes surgical repair of the diaphragm after hemodynamic stability is achieved, gentle ventilation, inhaled nitric oxide, and ECMO in selected cases. Furthermore, echocardiography is considered as an important tool to assess right heart function and evaluate the degree of pulmonary hypertension non-invasively in newborns. The presence of pulmonary hypertension is estimated based on peak tricuspid regurgitant velocity and systolic artery pressure. Another supportive care measure is oxygen therapy which acts as a double-edged sword with beneficial effects through vasodilatory activity in the lung and negative effects by the production of high concentrations of free radicals [15] [94]. Another available option is vasodilator drugs therapy such as Sildenafil or Bosentan. Sildenafil blocks phosphodiesterase 5 and demonstrates antiproliferative effects through the cGMP pathway [89]. Bosentan is an endothelin A and B receptor antagonist that reduces the vasoconstriction effects of the endothelin-1 pathway in CDH neonates.

1.9 MicroRNAs in pulmonary hypertension

MicroRNAs are an important class of post-transcriptional regulators playing a key role in the regulation of biological processes from developmental timing to apoptosis. These short (~22 nucleotides), non-coding RNAs inhibit translation of target mRNAs [95]. Synthesis of microRNAs is a two-step procedure initiated in the nucleus and then continued in the cytoplasm. First, the transcription of primary (pri)-miRNA proceeds by RNA polymerase II in the nucleus. Then pri-miRNA is processed to hair-pin structures, known as precursor miRNA (pre-miRNA), by a large protein complex. This complex is called the microprocessor and consists of Drosha (ribonuclease III) and Pasha (DGCR8). Pasha is a double stranded RNA-binding protein, recognizing the cleavage site. Pre-miRNA is then exported to the cytoplasm via exportin-5 to be cleaved by DICER (ribonuclease III) into double-stranded miRNA/miRNA molecules. The miRNA duplex is separated into two single strands: 1) the guide strand or mature miRNA which interacts with complementary sequences in target mRNA and promotes inhibition or degradation; 2) the passenger strand which is usually degraded. This process is possible when miRNA duplex loads on the RNA-induced silencing complex (RISC) containing argonaute and other proteins [96] (Figure 1).

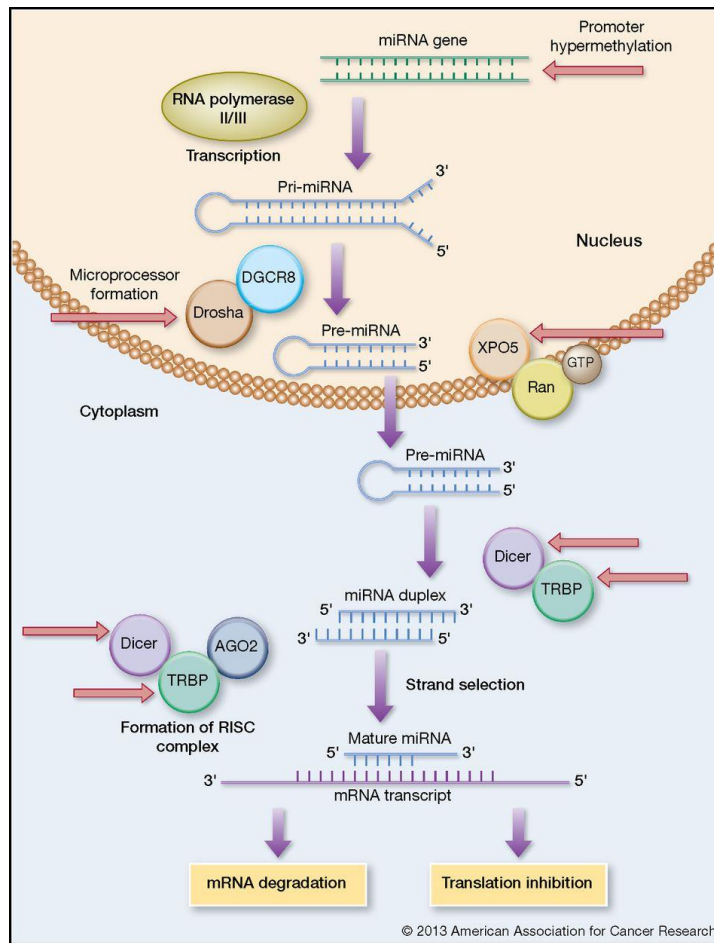


Figure 2. MicroRNA biosynthesis and mechanism of action: miRNA is transcribed to pri-miRNA by RNA pol II in the nucleus followed by cleavage to pre-miRNA by the Drosha/DGCR8 complex protein. Next, the pre-miRNA is exported to the cytoplasm by Exportin-5 through the nuclear pore. In the cytoplasm, pre-miRNA undergoes more processing by the activity of ribonuclease Dicer into a miRNA duplex. Finally, miRNA binds to the RISC complex and splits into a miRNA guide strand and complementary miRNA. MiRNA leads to degradation or translational inhibition of target mRNAs while complementary miRNA is usually degraded. (Mulrane *et al.* 2013, used with permission of the journal).

Recent studies have shown that microRNAs have a role in the pathogenesis of pulmonary hypertension and pulmonary dysfunction [97] [98]. Circulating microRNAs let-7b/c, miR-1307,

-8084, -185, -210, and -331 are downregulated in CDH infants with developed chronic lung disease and pulmonary hypertension. These 7 microRNAs have a role in molecular pathways related to cell cycle regulation, tissue homeostasis and morphogenesis [97]. Moreover, miR-21 expression is downregulated in both induced pulmonary hypertension rat model and human patients with pulmonary arterial hypertension, leading to the proliferation of vascular smooth muscle cells and vascular remodeling through the BMP signaling pathway. MiR-126 contributes to angiogenesis and vascular integrity [99]. Deletion of miR-126 can cause impaired vascular development and endothelial cells dysfunction due to an inappropriate response to the VEGF and FGF signaling pathways [100].

1.9.1 The relationship between the miR-200b and VEGF signaling pathway

In this project, I study the interaction between miR-200b and the VEGF signaling pathway because VEGF-A and its receptors are targets of miR-200b. Also, VEGF plays a critical role in the angiogenesis and it is mainly involved in pulmonary hypertension. MiR-200b is a member of the miR-200 family, known as a regulator of epithelial-to-mesenchymal transition (EMT) in cancer and an anti-angiogenic factor by targeting angiogenic cytokines and transcription factors [101]. A previous study has revealed that transfection of the human A549 cell line (lung carcinoma) with miR-200b reduces protein levels of VEGF, VEGFR-1, and VEGFR-2 suggesting that miR-200b may have a therapeutic potential in cancer as an angiogenesis inhibitor. MiR-200b targets VEGF and its receptors in human microvascular endothelial cells and impedes their angiogenic response to migrate and form vessel tubes in Matrigel [102]. Also, intravitreal injection of miR-200b mimics in the retina of diabetic rats prevents overexpression of

VEGF at both the mRNA and protein levels [103]. In lung adenocarcinoma cells derived from *Kras/ p53*-mutant mice, a negative correlation is noted between miR-200b and VEGFR-1 owing to suppression of invasion and metastasis [104].

1.10 Preliminary data

Dr. Keijzer's research group has focused on the role of microRNAs (specifically miR-200b) in abnormal lung development and the pathogenesis of CDH. We previously reported that from 319 microRNAs, miR-10a and miR-200b expression are increased in human CDH lungs compared with age-matched controls (8). Interestingly, infants with severe CDH surviving FETO demonstrate higher expression of miR-200b in their tracheal fluid after FETO. In the nitrofen-induced CDH rat model, pulmonary hypoplasia was associated with downregulation of miR-200b at embryonic day (E) 13,15 and 18 and upregulation at E21. Prenatal miR-200b therapy in pregnant rats improved lung hypoplasia and decreased the incidence of CDH in pups [105].

To better understand the role of miR-200b in the development of normal and abnormal lung development, our laboratory created a miR-200b knockout (KO) mouse model. Our data indicated that miR-200b KO mice have abnormal lung development due to a decrease in distal airway branching, thicker alveolar walls, dysfunctional surfactant, and higher expression of Twist (a marker for EMT) [18]. After extensive studies on the role of miR-200b in lung development and pulmonary hypoplasia associated with CDH, we have shifted our focus to investigate whether miR-200b has a role in pulmonary hypertension. We performed echocardiography to assess changes in the right heart size and function. Our preliminary results showed that miR-200b KO mice display a 14% increase in cardiac output and a shortened

pulmonary acceleration (PAT) time by 24%. PAT represents pulmonary artery pressure in mice and is defined as a time when blood flows into the pulmonary artery to get the peak of velocity. Furthermore, KO mice have cardiac hypertrophy resulting from an increase in the right ventricular internal diameter during systole and diastole. These preliminary results encouraged us to investigate the role of miR-200b in pulmonary hypertension, the main cause of mortality in CDH and other cardio-pulmonary developmental diseases.

1.11 Hypothesis and objectives

We hypothesized that miR-200b knockout causes vascular changes that result in pulmonary hypertension. To test our hypothesis, we defined the following objectives:

- 1) To evaluate morphological pulmonary vasculature changes in miR-200b KO mice
- 2) To determine the role of miR-200b in pulmonary hypertension by targeting of the VEGF signaling pathway

CHAPTER 2. MATERIALS AND METHODS

2.1 Creation and validation of miR-200b KO mice

Ethical approval was obtained from the Animal Care Committee at the University of Manitoba for all animal experiments [protocol number: 16-046 (AC11402)].

Dr. Keijzer's laboratory previously created miR-200b KO mice [18]. Briefly, the miR-200b gene (wildtype allele) was deleted from embryonic stem cells in C57Bl/6N mice and replaced by a NorCOMM cassette using homologous recombination. The cassette contained loxP sites that were recognized and excised by the Cre-recombinase enzyme. Validation of the KO mice was done by PCR, DNA sequencing and Southern blot analysis. The KO line was maintained through selective breeding of miR-200b heterozygous mice. The heterozygous, homozygous and wildtype (WT) pups were genotyped using PCR from an ear biopsy. First, DNA was extracted from ear samples using a DNA extraction kit (Quantabio). Then, PCR was performed using the following reverse and forward primers (Table 1).

Table 1. List of primers used for genotyping of miR-200b KO mice

Primers sequences	Primer type
GH717: 5'-CACCGACGCCAATCACAAACAC-3'	Reverse
GH3369: 5'-AGGGGAACTTGTCTATGGCCATG-3'	Forward
GH3370: 5'-CCCATAGCCCTACCTTGGATAAGG-3'	Reverse

Initial denaturation occurred at 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds. Then, the final elongation was performed at 72°C for 5 minutes and the temperature was held at 10°C. Primers GH 3369 and GH 3370 detected the 494 bp band in the WT allele while primers GH3369 and GH717 detected a shorter fragment of 390 bp in the mutant allele.

2.2 Lung fixation

8-week-old miR-200b KO and WT male mice (n=4) were sacrificed per our protocol. The chest cavity was partially opened by cutting the diaphragm. Then, the trachea was cut, and a 21-gauge blunt needle inserted into the trachea and tied with thread. The needle was connected to a reservoir containing 10% formalin with 25 cm H₂O pressure. The stopcock was opened, and the lung was inflated with formalin for 2 minutes. After lung inflation, the trachea was tied off and the thread and needle were removed. The inflated lung was carefully extracted and fixed in 10% formalin on a shaker at 4°C overnight. Next, tissues were processed in a graded series of ethanol (75%, 95%, and 100%) and xylene. Then, left and right lungs were embedded in paraffin separately and 5 µm sections were cut using a microtome for sectioning. Only the left lung was used for histology, including Verhoeff-Van Gieson's (VVG) staining and immunohistochemistry (IHC).

2.3 Verhoeff Van Gieson's staining

Pulmonary hypertension is characterized by an increase in the thickness and stiffness of the medial and adventitial layers of the vessels. VVG staining was used to evaluate the structural

changes in the lung vessels. The Verhoeff stain component has iron-hematoxylin that specifically binds to elastic fibers. The Van Gieson stain component is comprised of picric acid and acid fuchsin that strongly bind to collagen fibers.

The paraffin embedded left lung of KO and WT mice (n=4) were incubated at 58°C for 30 minutes followed by rehydration in an alcohol series and deparaffinization in xylene. Then, slides were stained with Verhoeff's solution containing 5% Alcoholic Hematoxylin, 10% Aqueous Ferric Chloride and Iodine solution for 1 hour. Slides were rinsed quickly with 2-3 changes of tap water and developed in 2% ferric chloride for 1-2 minutes. Then, development was stopped with several washes in tap water and slides were checked under the microscope for black elastin fibers. Slides were washed in tap water and treated with solution D containing 5% sodium thiosulfate to remove excess iodine followed by washing with tap water for 5 minutes. Lastly, samples were counterstained in Counterstain I (Van Gieson stain) for 5 minutes and dehydrated in a graded series of alcohol, cleared in xylene and coverslipped with mounting medium.

For vascular remodeling assessment, all available arteries (approximately 34 vessels per mouse) from the VVG stained sections of the left lung were analyzed using Image J software with 40X magnification. Only arteries were included in the assessment if the maximal external diameter did not exceed the minimal diameter by more than 50%. The following parameters were calculated in the miR-200b KO and WT groups (n=4): external diameter (ED), internal diameter (ID) and adventitial diameter (AD). Then, based on the calculated diameters, the following parameters were calculated: arterial wall thickness = $(ED-ID)/2$; the percentage of medial wall thickness: $MT = (ED - ID)/ED*100$; the percentage of adventitial thickness: $AT = (AD - ED)/ED*100$ [106].

2.4 Micro-CT scan

A major limitation in studying changes in pulmonary vascular disease such as pulmonary hypertension is the lack of proper morphometric quantitative methods [107] [108]. While histologic assessment cannot represent the full complexity of the pulmonary vasculature, Micro-computed tomography (micro-CT) allows one to determine vascular branching, volume, diameter, length, and density or even vessel generation in the entire lung [107]. This approach provides high resolution, quantitative, three -dimensional (3D) images to visualize vascular remodeling in small animal models [109]. To date, only a few studies have demonstrated the full complexity of vessel branching in small animals particularly mice to the microlevel [107].

2.4.1 Surgical procedure

KO and WT mice were anesthetized with 5% isoflurane for 10 minutes per our approved animal use protocol. A deep anesthesia state of animals was verified. Then, the animal was pinned supine to the dissection board (Styrofoam), and an anesthesia tube was placed into the mouse's nose to provide continues anesthesia during the procedure. The inferior vena cava (IVC) and descending aorta near the kidneys were cut to exsanguinate the mouse. Next, the chest cavity was opened, and a 21-gauge winged infusion needle was placed into the right ventricle, stabilized with glue and connected to a 3-way stopcock and 5 ml syringe (Figure 2).

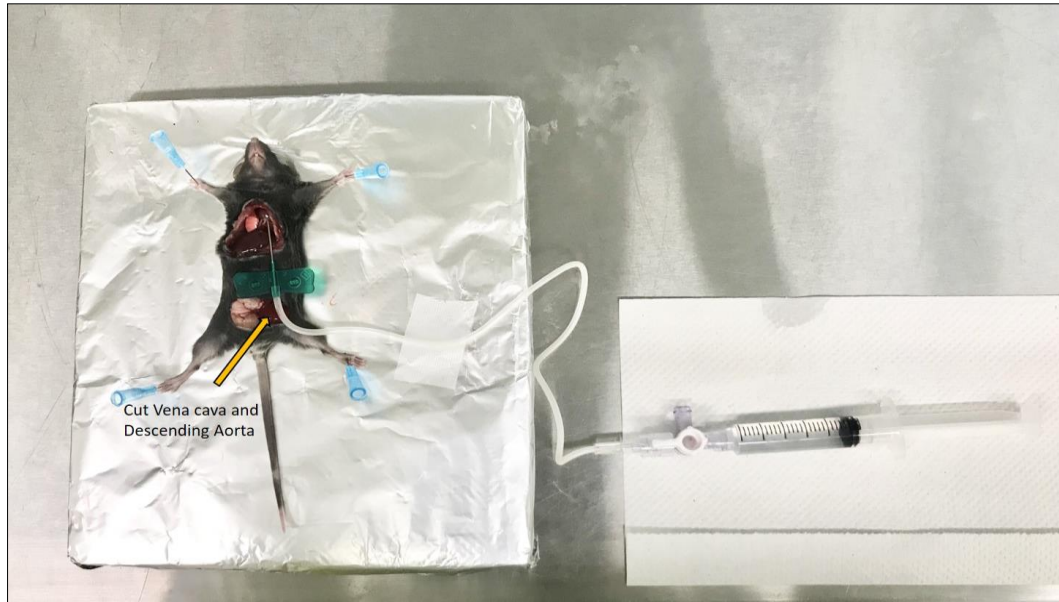


Figure 3. Animal preparation for cardiac perfusion. Anesthetized animal was placed on a dissection board and the inferior vena cava and descending aorta were cut to exsanguinate the mouse. The diaphragm and ribs were cut to expose the heart. A 21-gauge winged infusion needle was fixed in the right heart to facilitate perfusion. Then, a butterfly catheter was attached to a 3-way stopcock and a syringe filled with 5 ml PBS.

The pulmonary circulation was immediately perfused with 5 ml phosphate buffered saline (PBS) via the right ventricle of the heart. The blood in the pulmonary vasculature was drained out through the vena cava and descending aorta and replaced with PBS. The stabilized winged infusion set attached to the 3-way stopcock prevented the production of air bubbles and manipulation of lung vessels during perfusion and ultimately resulted in better CT images. After good perfusion, the lungs became a light pink color (Figure 3).

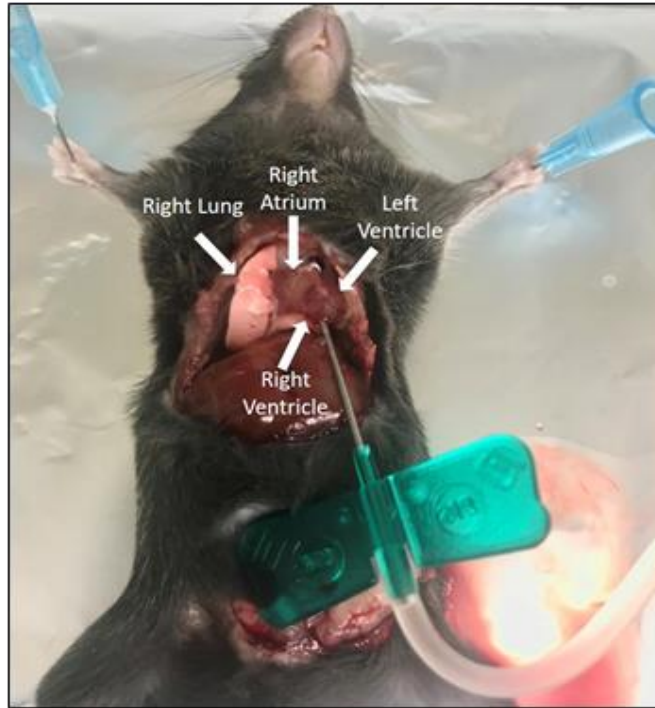


Figure 4. Perfusion of pulmonary circulation via right ventricle. Successful perfusion results in blood exiting from the cut vessels (IVC/aorta) and turning the color of the lungs to a light pink.

2.4.2 Animal preparation for Micro-CT imaging

A radio-opaque silicon rubber reagent called Microfil (Flow Tech, Carver, MA) was used to enhance the contrast during CT scanning. The most prominent feature of Microfil making it an appropriate choice for vascular imaging is its minimal volume change in the vessels. To prepare Microfil solution, 2 ml MV-Compound, a yellow component of the Microfil kit, was mixed with 2.5 ml MV-Diluent. Then 450 μ l catalyzer reagent was added to the mixture to facilitate polymerization. Microfil solution was loaded into the 5 ml syringe attached to the 3-way stopcock and was perfused very slowly into the pulmonary circulation. Microfil is a viscous reagent therefore, gentle perfusion is necessary to avoid the risk of vascular rupture. After

injection, the vena cava and descending aorta were quickly ligated using micro-clip and clip forceps (Roboz Surgical Instruments, cat. no. RS-5431 and RS-5440) to prevent leakage of Microfil. The Microfil was allowed to solidify at room temperature for 90 minutes. Then the lung tissue was dissected carefully and fixed in 10% formalin. Before placing the sample into the micro-CT chamber, the lung was soaked in paraffin to prevent it from drying out.

Lungs were scanned for 45 minutes using the Skyscan 1176 x-ray microtomography system, at 9 μm resolution. The micro-CT system was adjusted to 650 ms exposure time, 0.3° rotation step, 90 kV and 160 μA with a 0.5 mm aluminum filter. Two-dimensional images were reconstructed from the raw data using NRecon (Bruker MicroCT, Kontich, Belgium). Reconstructed images were processed and analyzed using CTan (Bruker MicroCT, Kontich, Belgium). The lung tissue was traced as a region of interest (ROI), the background excluded, and images within the region of interest were saved in a new folder. The reduced data set was analyzed in CTan software to calculate the following parameters in 3D images: Total lung volume, the ratio of vascular volume to total lung tissue volume (mm^3/mm^3) and the ratio of vascular surface area to total vascular volume. The images were binarized by setting the lower threshold around 60 and an upper threshold of 255. Shrink-wrap was performed in 3D space to create an ROI by adapting to the shape of the lung. Then, the images were de-speckled to eliminate the white and black speckles less than 50 voxels. The CTVox (Bruker MicroCT, Kontich, Belgium) was used to generate 3D images from the lung vasculature.

2.5 Immunohistochemistry (IHC)

IHC was used to determine the expression and localization of VEGFR-1 and VEGF-A proteins. Paraffin embedded left lungs of miR-200b KO and WT mice (n=4) were dewaxed and re-hydrated using a graded ethanol series. Then, citrate buffer from Vector Laboratories (H-3300) was used in a boiling water bath for 30 minutes to relax protein structures in the samples and unmask antigenic sites for the antibody. Slides were cooled off at room temperature for 20 minutes and washed 3 times in PBS 1X. Sections were treated with a blocking solution containing FBS, 10% Roche Blocking Reagent in Maleic Acid Buffer and 10% Tween-20 in PBS for 30 min at room temperature to eliminate background signal. After blocking, 3% H₂O₂ was applied for 10 min to remove endogenous hydrogen peroxidase. Avidin and Biotin proteins were blocked using the Avidin/Biotin blocking solution (Vector sp-2001). Then, tissues were incubated with primary antibodies in blocking solution overnight at 4°C. The rabbit monoclonal anti-VEGF Receptor 1 antibody (Abcam; ab32152, 1:200), the rabbit polyclonal anti-VEGF Receptor 2 antibody (Abcam; ab2349, 1:100), and the rabbit monoclonal anti-VEGFA antibody (Abcam; ab232858, 1:500) were used as primary antibodies.

On the second day, tissues were incubated with a goat anti-rabbit biotinylated secondary antibody diluted 1:400 in blocking buffer for 30 min at room temperature. ABC solution (Vector PL-6100) was used to bind to the biotin-labeled secondary antibody. ImmPACT DAB substrate (Vector Laboratories) was used to show the expression of the protein of interest as a brown color. Sections were immersed in double-distilled water and counterstained with Mayer Hematoxylin (Vector H-3404). Finally, slides were immersed in 2% sodium bicarbonate solution for 20 seconds, dehydrated in a graded series of alcohol, cleared in xylene and coverslipped with mounting medium.

2.6 Western blotting

The left lung of 8-week-old miR-200b KO and WT mice were dissected, frozen in liquid nitrogen and homogenized in lysis buffer. RIPA lysis buffer comprised of 150 mM sodium chloride, 50 mM Tris (pH 8.0), 0.1% SDS, 1.0% NP-40, 0.5% sodium deoxycholate, and 2 mM EDTA (pH 8.0). For homogenization, 10 μ l of lysis buffer was added per mg of tissue weight. Protease inhibitor tablet (Roche complete-mini) and phosphatase inhibitors (0.1 M sodium orthovanadate, 0.1 M sodium pyrophosphate, 0.1 M EGTA, and 1 M sodium fluoride) were added to the RIPA buffer. Protein extraction was completed by sonicating the tissues for a total of 30 seconds. The Bio-Rad RC DC Protein Assay was used to measure protein concentration. A 9% acrylamide loading gel and 4% acrylamide stacking gel were prepared and following protein denaturation, 40 μ g of total protein were loaded on SDS-PAGE and transferred over 1 hour to the polyvinylidene difluoride membrane. The membrane was washed with TBS for 10 min and blocked in a 5% skim milk solution for 2 hours at room temperature, then incubated with the primary antibody (rabbit monoclonal anti-VEGF Receptor 1, Abcam; ab32152, 1:1000) in 1% skim milk overnight at 4°C.

On the second day, the membrane was washed 3 times with TBS-T and incubated with a horseradish peroxidase conjugated goat-anti-rabbit secondary antibody (1:10000) (Cat. No. 1706515, Bio-Rad Laboratories) for 1.5 hours. The membrane was visualized using an enhanced chemiluminescent substrate (Bio-Rad) and scanned using trans-illuminator (ChemiDoc™ System). Band densities were normalized to the respective α -tubulin signal (Cell Signaling; # 2124, 1:1000) as a loading control. The membrane was incubated with the α -tubulin antibody in 5% BSA, at 4°C with gentle shaking, overnight.

2.7 Analysis and statistics

All data were presented as the mean $\pm$ standard error of the mean. For comparing two groups, as the data was not normally distributed, Mann-Whitney U test was used. For comparing more than 2 groups one-way, an ANOVA with Tukey-Kramer multiple comparisons test was used. All graphs were created using GraphPad-Prism, version 8. P values ≤ 0.05 were considered significant.

CHAPTER 3. RESULTS

3.1 Morphometric assessment of pulmonary arteries

I used VVG staining to study vascular remodeling in the pulmonary vessels of 8-week-old miR-200b KO mice and compare the results with the WT group. With VVG staining, collagen fibers stain pink to red in the adventitia, elastic fibers stain dark blue to black in the media and other tissue components stain yellow (Figure 5).

Measurement of morphometric parameters showed that miR-200b KO mice have 35% increased arterial wall thickness (Figure 6A). Also, the degree of medial wall thickness and adventitial wall thickness in the pulmonary arteries and arterioles of miR-200b KO mice were significantly higher (Figure 6B & C) ($p < 0.0001$). Histologic sections demonstrated that arteries of miR-200b KO mice have a thicker medial and adventitial wall when compared to WT lungs (Figure 7). To determine whether vascular remodeling in the pulmonary vessels is related to the size of the vessels, I categorized vessels into five groups based on the external diameter: less than 10 μm , 10 to 20 μm , 20 to 40 μm , 40 to 60 μm and greater than 60 μm . The arterial wall thickness was significantly higher in the medium-sized lung arteries of the miR-200b KO mice with an external diameter between 10 to 20 μm and 20 to 40 μm ($P < 0.001$) (Figure 8). In miR-200b KO mice, the percentage of medial wall thickness showed a significant difference in small vessels with an external diameter less than 20 μm ($P < 0.05$) Interestingly, the most significant changes were observed the in small vessels with an external diameter between 10 to 20 μm ($P < 0.001$). Also, the medial wall thickness % of lung vessels in miR-200b KO mice with an external diameter between 20 to 40 μm showed a significant increase ($P < 0.01$) (Figure 9). For the degree of adventitial wall thickness, the most significant increase was observed in small-sized arteries with

an external diameter less than 10 μm and between 10 to 20 μm in the miR-200b KO group ($P < 0.05$) (Figure 10).

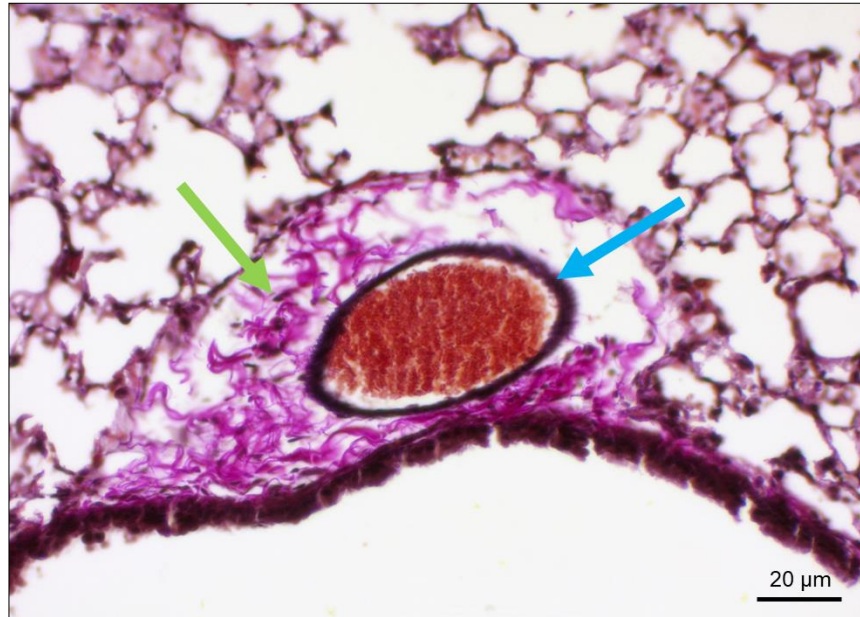
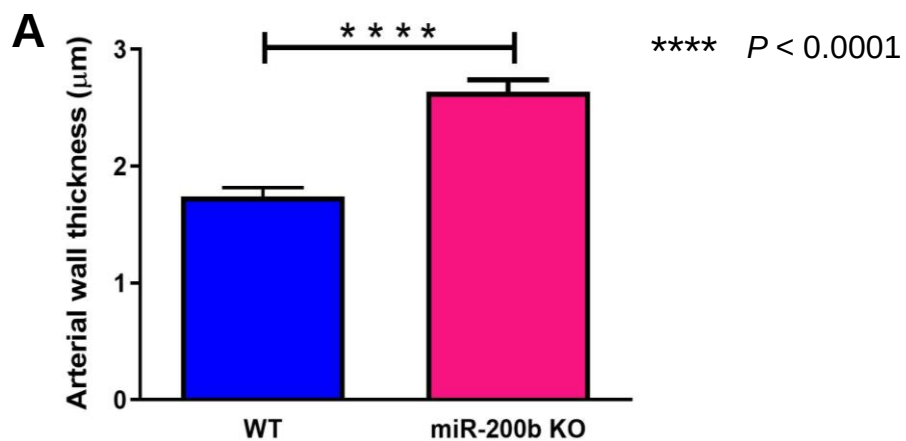


Figure 5. Typical view of an artery stained with VVG staining. VVG staining elucidates the differences between elastic and collagen fibers from other tissue components. Collagen fibers in the adventitia stain pink to red (green arrow) and elastin fibers stain dark blue to black (blue arrow) in the mouse lung tissue. In this section, the lumen of the vessel is filled with RBCs. Image obtained with 20X magnification.



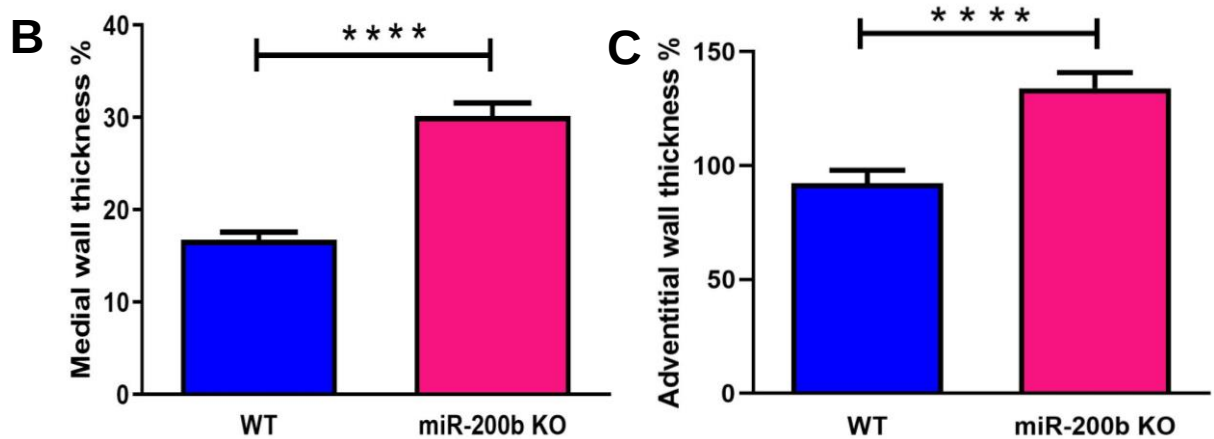


Figure 6. MiR-200b KO mice have increased arterial, medial and adventitial wall thickness in the pulmonary vessels. Arterial wall thickness is significantly increased in miR-200b KO mice compared with WT group (A). In addition, miR-200b KO mice have an increase of 47% medial wall thickness (B) and 32% adventitial wall thickness (C) ($P < 0.0001$) (n=4).

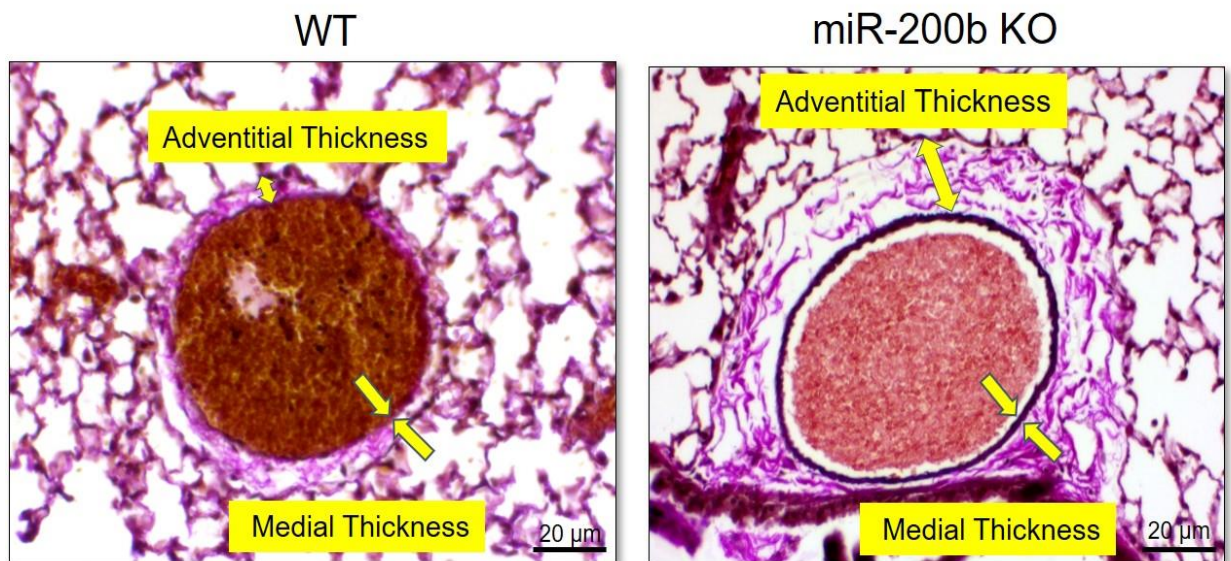


Figure 7. The adventitial and medial vessel wall are thicker in pulmonary arteries from miR-200b compared with WT mice. VVG staining shows thicker adventitia and media layers of the arteries in lung sections from miR-200b KO 8-week-old mice compared with the WT group. Images obtained with 20X magnification.

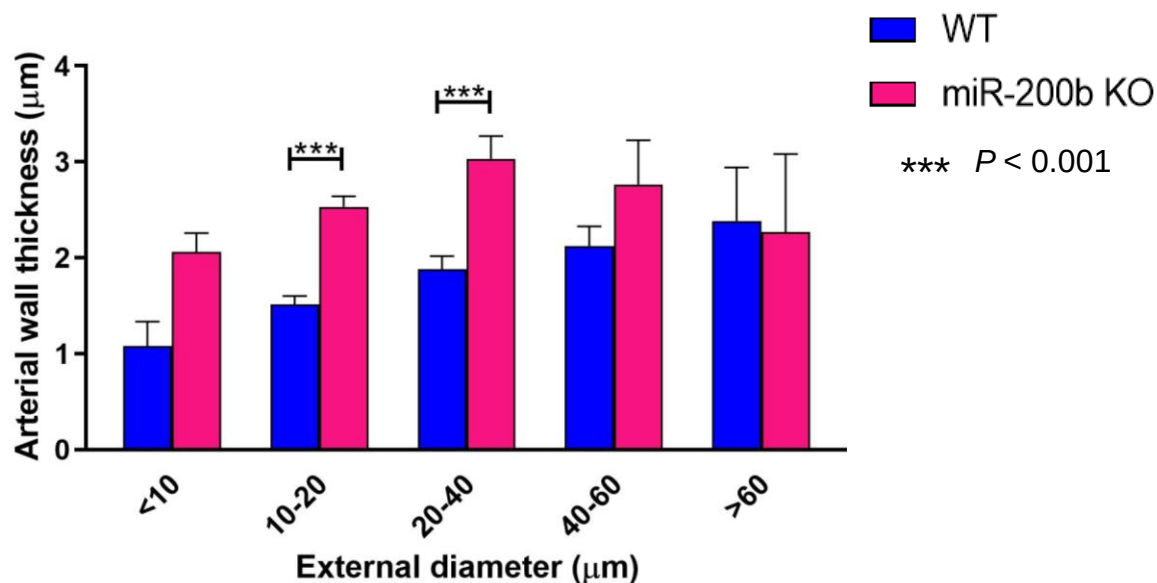


Figure 8. Medium-sized pulmonary arteries have thicker arterial walls based on different external diameter categories. Arterial wall thickness changes are observed in arteries with an external diameter between 10 to 40 μm in the miR-200b KO group ($P < 0.001$) (n=4).

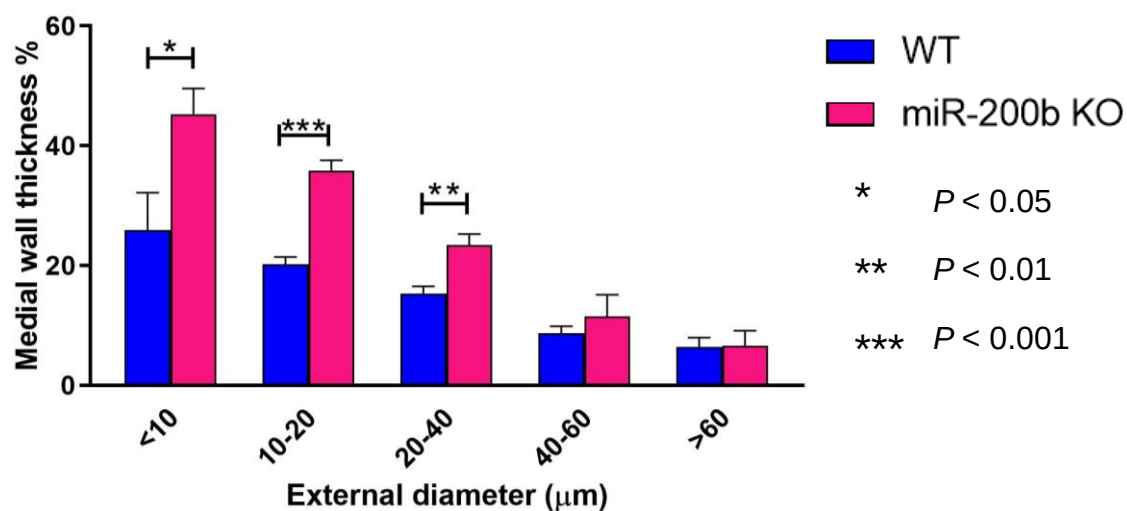


Figure 9. Small-sized pulmonary arteries have a thicker medial wall based on different external diameter categories. In miR-200b null mice the highest increase in the percentage of medial wall thickness occurs in 3 different groups of vessels with an external diameter less than 10 μm ($P < 0.05$), between 10 to 20 μm ($P < 0.001$) and 20 to 40 μm ($P < 0.01$) (n=4).

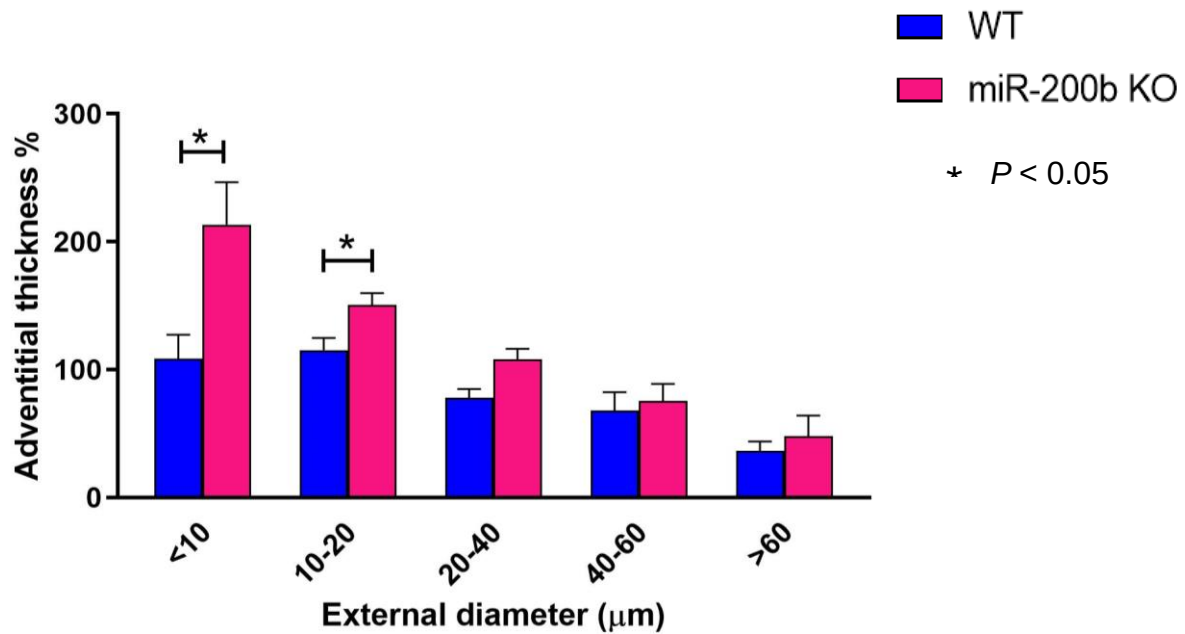


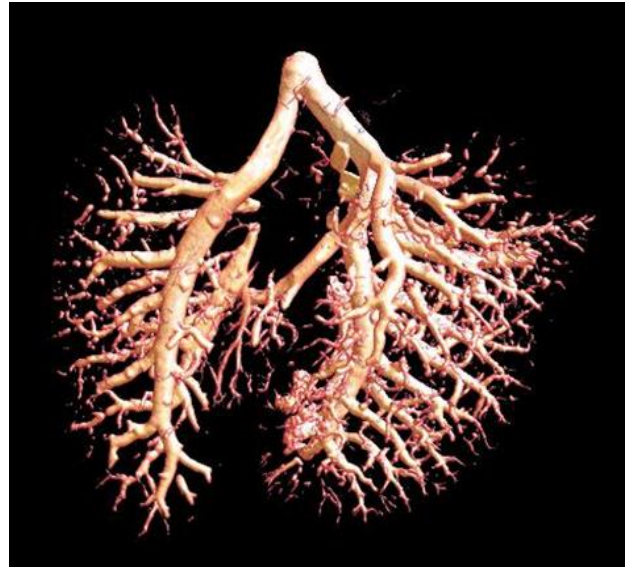
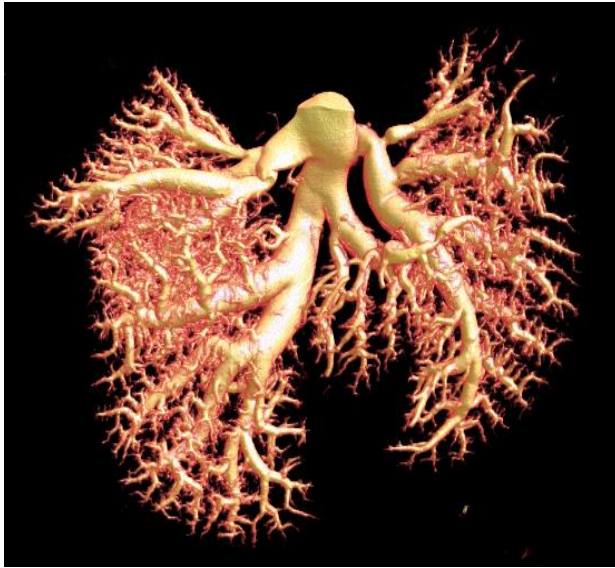
Figure 10. Small-sized pulmonary arteries have the thickest adventitial wall based on different external diameters categories. MiR-200b KO mice have increased adventitial wall thickness only in small-sized pulmonary arteries with an external diameter less than 20 μm ($P < 0.05$) ($n=4$).

3.2 Micro-CT analysis of the pulmonary vasculature

Four miR-200b KO mice and 3 WT mice underwent micro-CT scanning to characterize vascular remodeling using 3D images of the vasculature in the entire lung. Perfusion of contrast agent (Microfil) provided a clear visualization of the pulmonary vasculature to the microlevel (8 μ m) in the experimental and control groups. The lungs of miR-200b KO mice demonstrated a greater abundance of contrast agent particularly in distal vessels compared with WT (Figure 11). Using micro-CT scan data, morphometric parameters including total lung tissue volume (mm³), the ratio of vascular volume to total lung volume (mm³/mm³) and the ratio of vascular surface area to total vascular volume (1/mm) were calculated.

The total lung volume comprised of vessels and parenchymal tissue was not significantly different between groups (Figure 12). Also, the ratio of vascular volume to total lung volume that represented vascular density was not different between groups (Figure 13). No significant differences were observed for the ratio of vascular surface area to the total vascular volume between miR-200b KO and WT groups (Figure 14). This parameter increases in pulmonary hypertension as a result of vascular pruning and a loss of microvessels. To further explore the pulmonary vascular remodeling, changes in the vessel volume were evaluated relative to the vessel lumen diameter (mm). The vessel diameter spectrum was divided into seven groups including less than 0.25 mm, 0.25 to 0.5 mm, 0.5 to 0.75 mm, 0.75 to 1 mm, 1 to 1.25 mm and more than 1.25 mm. The average of vessel volume in different vessel diameter miR-200b KO group did not reach a significant difference by ANOVA test (Figure 15). All together, the micro-CT data did not reveal any significant differences between the pulmonary vasculature of the miR-200b KO and WT group.

WT



miR-200b KO

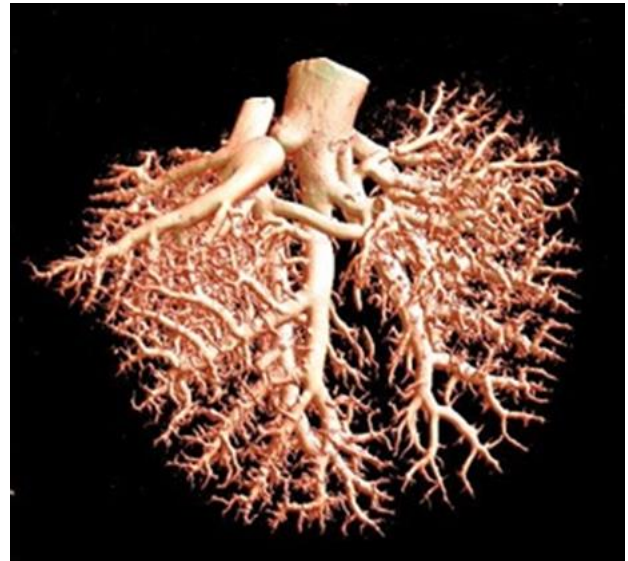
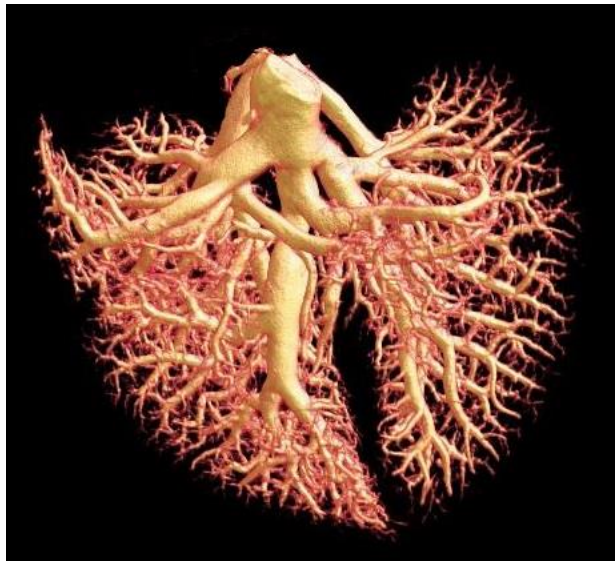


Figure 11. 3D image view of the pulmonary vasculature filled with contrast agent *microfil*. Micro-CT of the lung demonstrated the distribution and branching of the pulmonary vessels in the entire lung of miR-200b KO and WT mice.

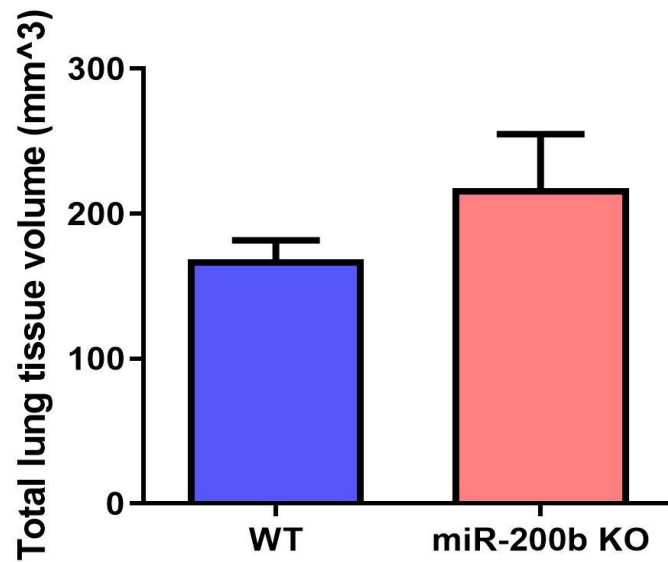


Figure 12. Total lung volume was not different in miR-200b KO and WT mice. Measurement of total lung volume (mm³) showed that there was no significant difference between groups (n=3).

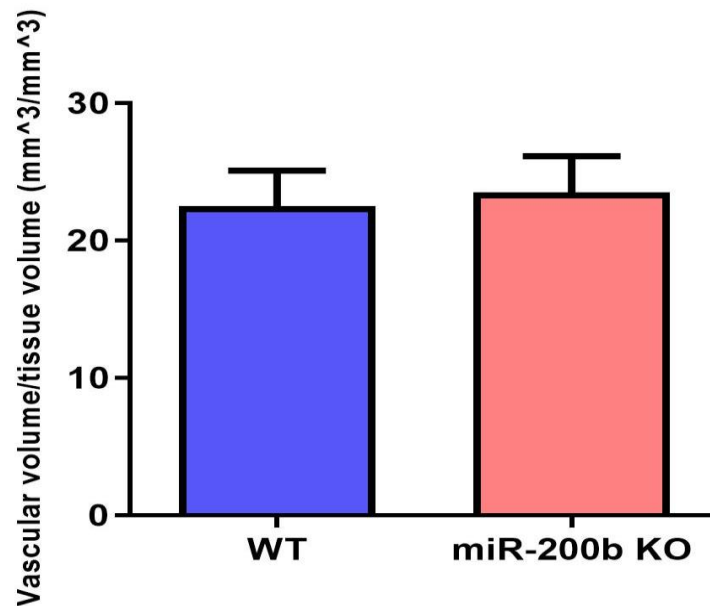


Figure 13. The vascular volume ratio to total tissue volume was not different in the miR-200b KO and WT group. This parameter demonstrated similar changes in both miR-200b KO and WT group, and no significant difference was observed (n=3).

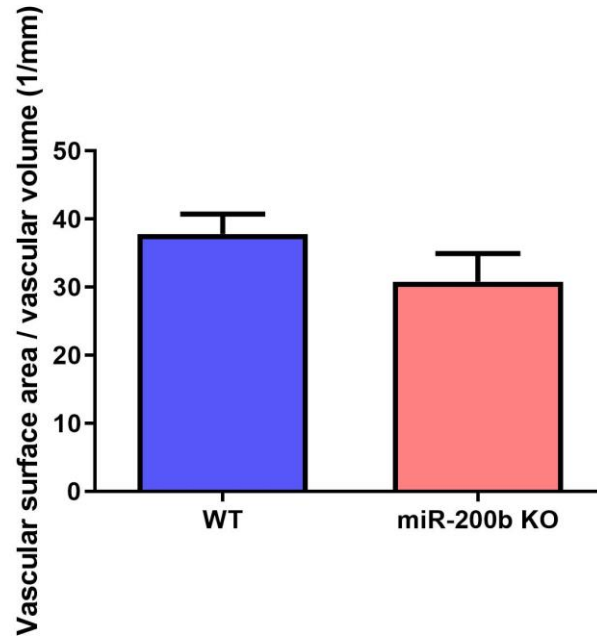


Figure 14. The vascular surface area ratio to vascular volume was not different in the miR-200b KO and WT group. The ratio of vascular surface area to vascular volume did not illustrate changes between groups (n=3).

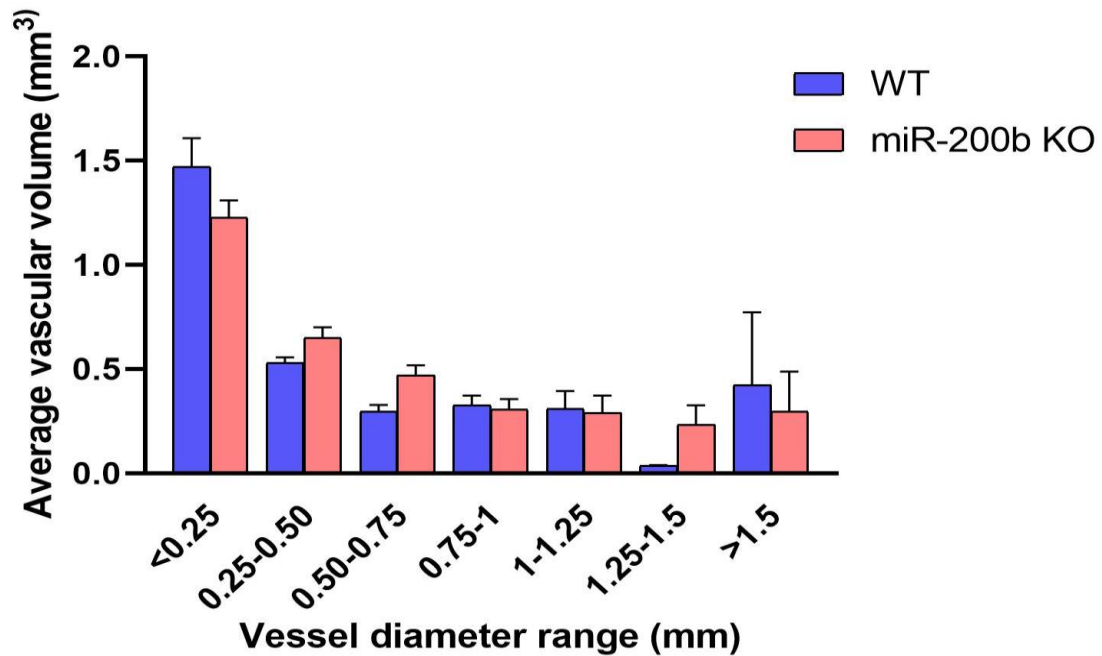


Figure 15. Histogram comparison of vascular volume ratio relative to vessel diameter in miR-200b KO and WT mice. The highest vascular volume was observed in vessels with a lumen diameter less than 0.5 mm in both miR-200b KO and WT group (n=3).

3.3 Expression of VEGF-A and its receptors VEGFR-1 and R-2 in the lung of miR-200b KO and WT mice

IHC was used to investigate the localization and expression of VEGF-A, VEGFR-1, and VEGFR-2. In all images, brown color represents the expression of the protein of interest. VEGF-A expression was detected in the alveoli, large and small bronchioles as well as the large arteries in the WT group. In this group, the highest expression of VEGF-A was observed in the epithelium of large bronchioles and vascular smooth muscle cells of the large pulmonary arteries. VEGF-A was down regulated in the lung of miR-200 KO mice and only expressed in the vascular smooth muscle cells of the large pulmonary arteries (Figure 16 & 17). Briefly, IHC analysis demonstrated that the overall expression of VEGF-A was less in the lung of miR-200b adult mice compared with WT.

WT mice displayed VEGFR-1 expression in the airways, large and small bronchioles and pulmonary arteries. However, signals were not very strong. Expression of VEGFR-1 was prominently higher in the miR-200b KO group as compared to age-matched controls in the WT group. VEGFR-1 was expressed in almost all tissues like bronchial epithelial cells, parenchyma and vascular smooth cells of arteries and veins in the miR-200b KO mice (Figure 18). No differences were observed for the expression of VEGFR-2 between miR-200b KO and WT lungs (Figure 19).

In summary, VEGF-A expression was downregulated and VEGFR-1 was upregulated in the lung of miR-200b null mice and VEGFR-2 expression did show any changes between the miR-200b KO and WT groups.

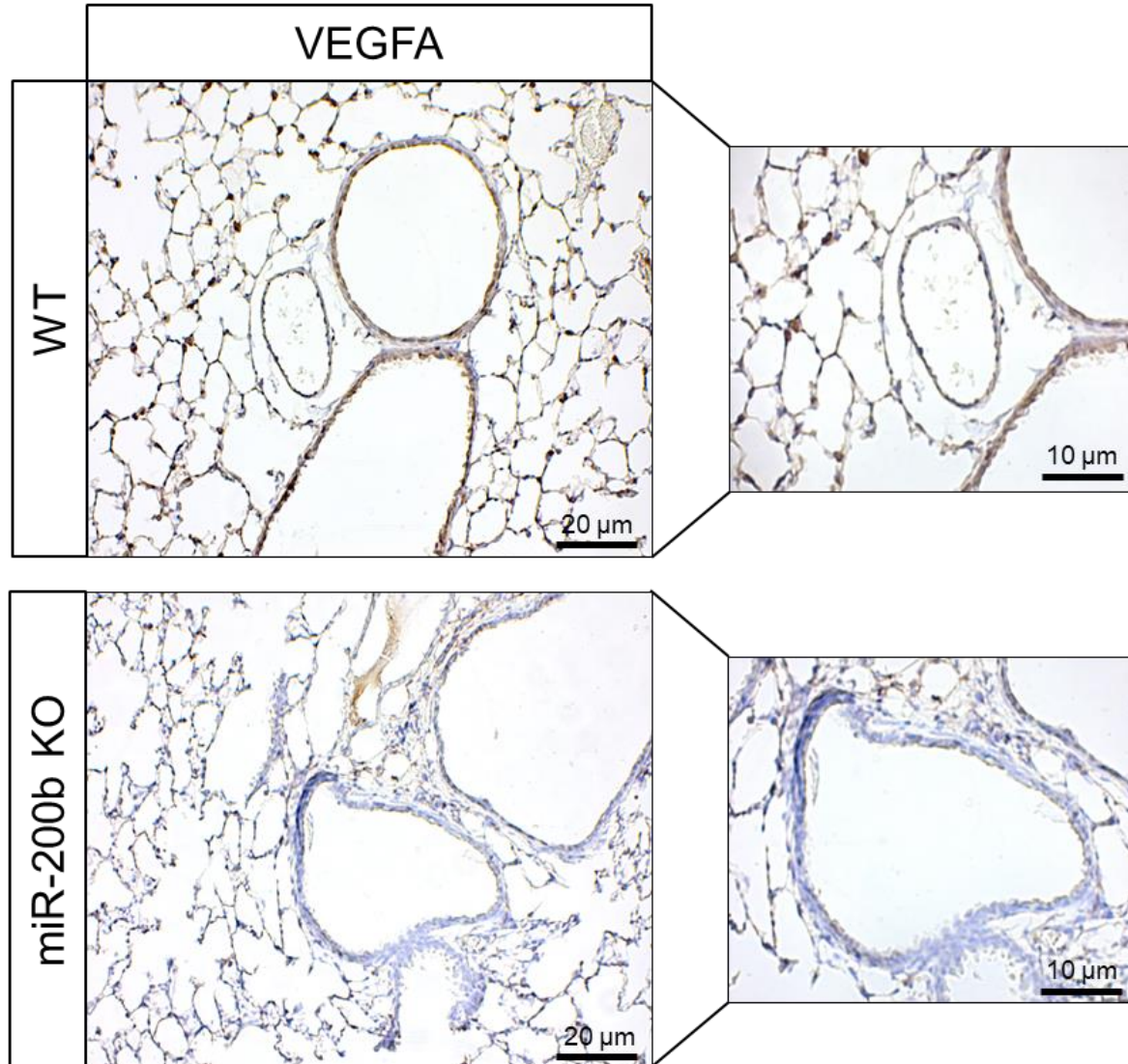


Figure 16. VEGF-A expression is lower in the airways and arterioles of miR-200b KO mice. Expression of VEGF-A was downregulated in the airways, bronchial epithelial cells and vascular smooth muscle cells of arterioles in miR-200b KO mice (n=4). Images obtained with 20X (left) and 40X (right) magnification.

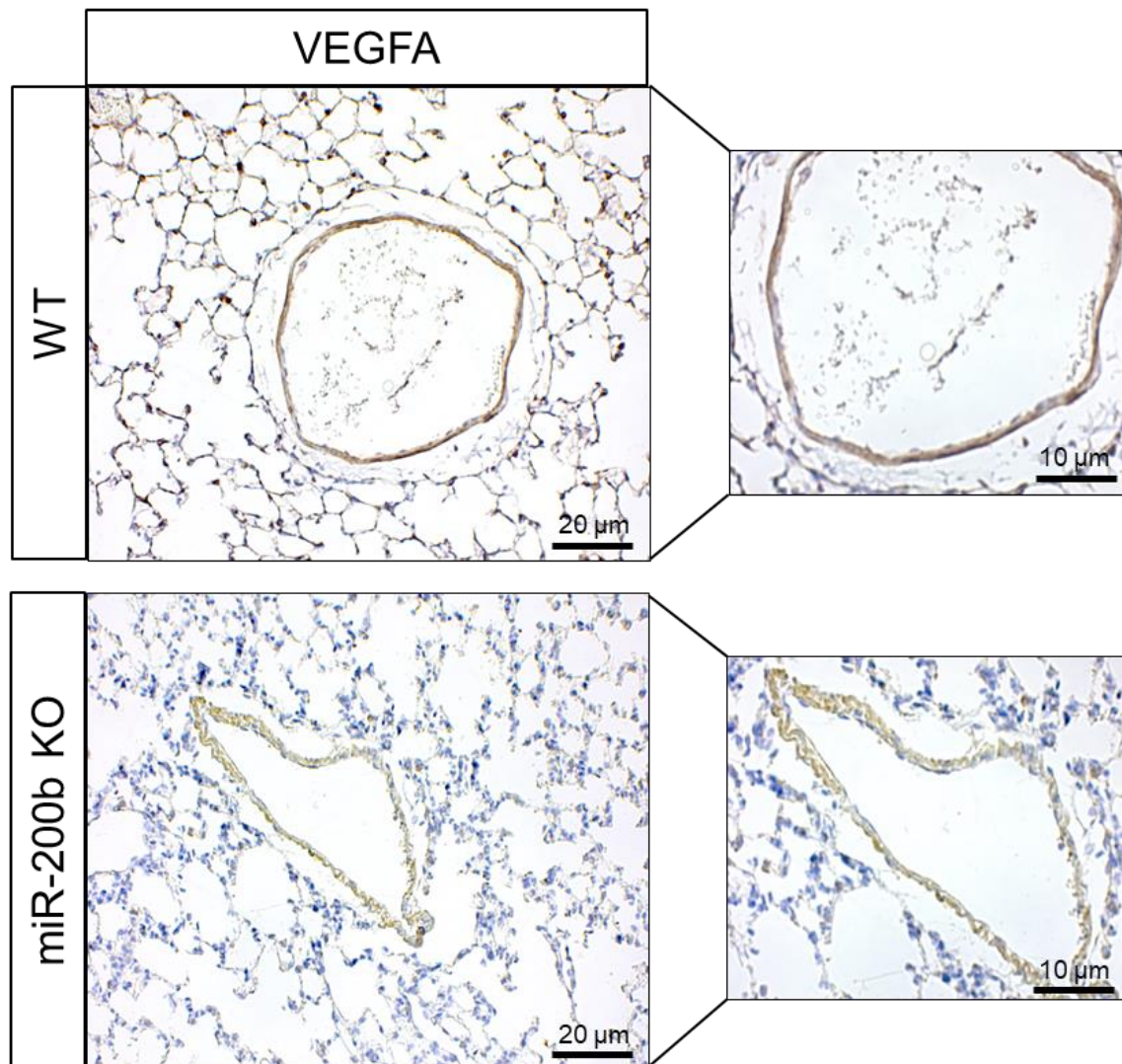


Figure 17. VEGF-A only expresses in the large pulmonary vessels of miR-200b KO mice. VEGF-A is downregulated in miR-200b KO group and only large pulmonary vessels have the expression of it (n=4). Images obtained with 20X (left) and 40X (right) magnification.

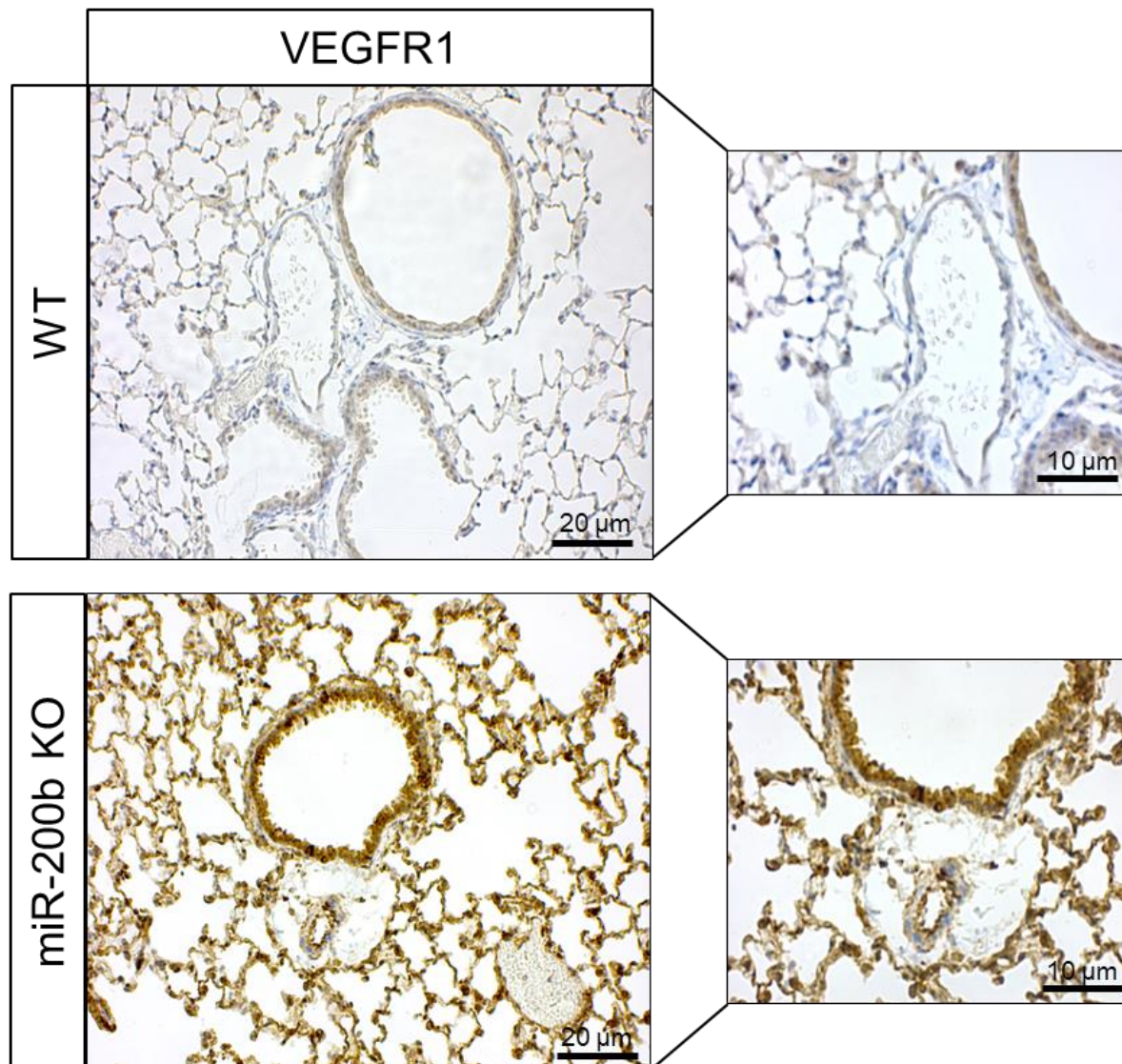


Figure 18. VEGFR-1 expression is higher in the bronchioles and arterioles of miR-200b KO mice. VEGFR-1 is upregulated in miR-200b null mice by obviously higher expression in the airways, bronchioles, and arteries (n=4). Images obtained with 20X (left) and 40X (right) magnification.

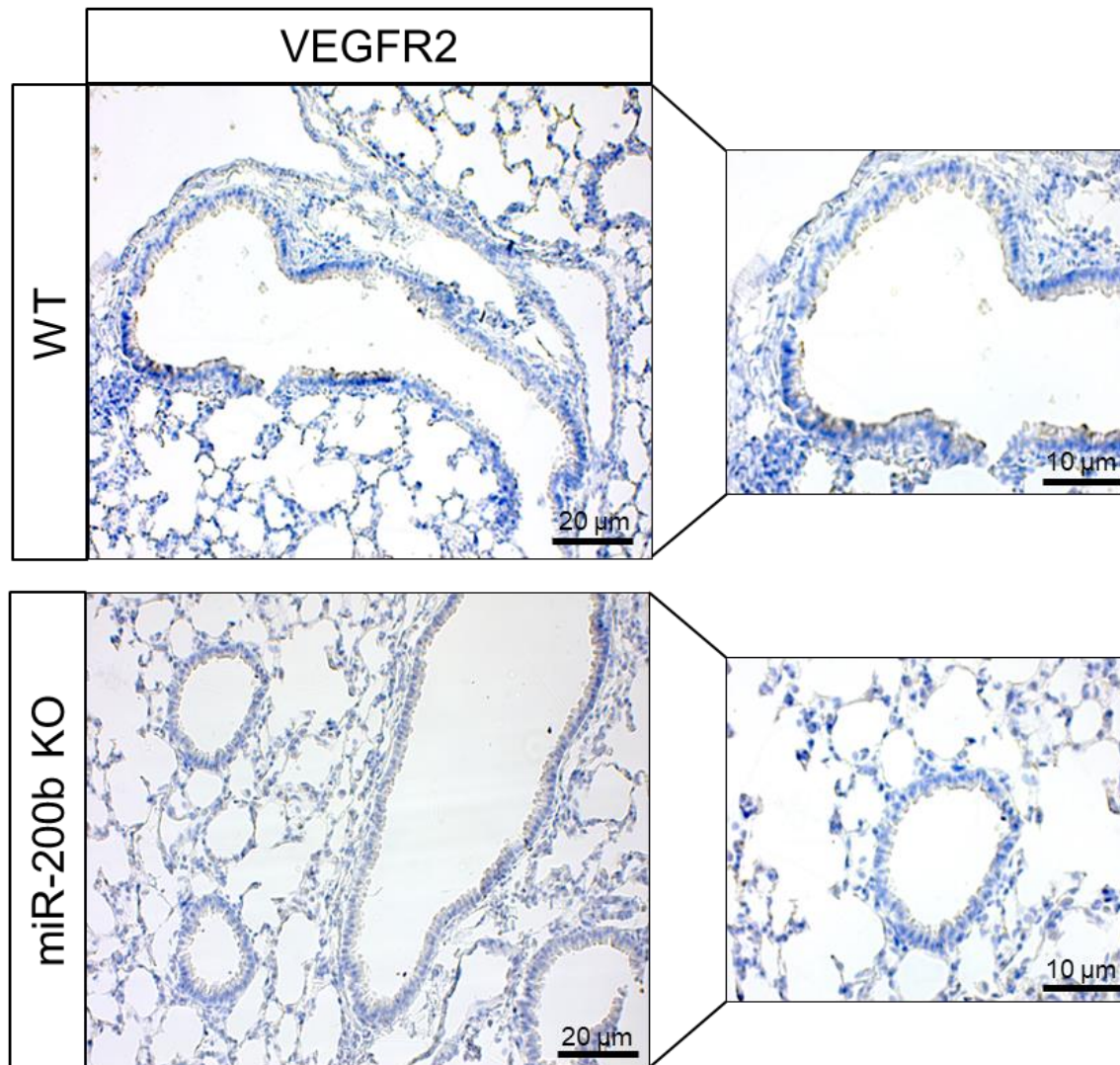


Figure 19. VEGFR-2 expression was not different in miR-200b KO and WT mice. Bronchioles, pulmonary vessels, and airways showed low expression of VEGFR-2 in the lung of miR-200b KO and WT mice (n=4). Images obtained by 20X (left) and 40X (right) magnification.

3.4 Quantitative evaluation of VEGFR-1 expression using Western blotting

Although IHC analysis showed upregulation of VEGFR-1 in the lungs of miR-200b KO mice, Western blotting showed no differences in normalized VEGFR-1 expression between miR-200b KO and WT mouse lungs. The estimated molecular weight for VEGFR-1 was 151 kDa and our Western blot result showed a band in the predicted range. Loading control Alpha Tubulin was used for normalization and detected with an anticipated ~ 50-kDa molecular weight (Figure 20).

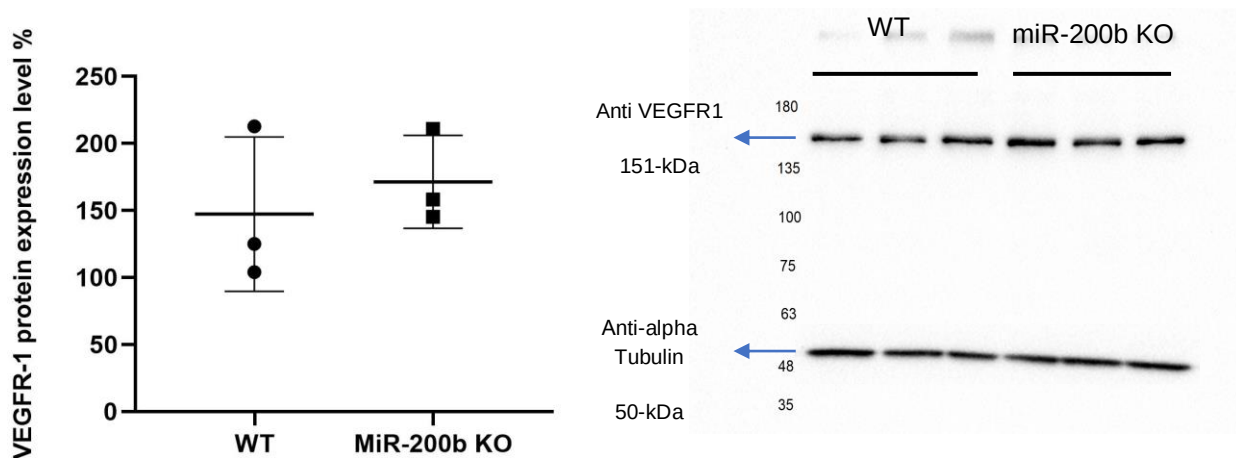


Figure 20. Western blotting showed no differences in VEGFR-1 in the lung tissues of miR-200b KO and WT mice. Western blot result for VEGFR-1 did not show any significant differences between miR-200b KO and WT groups. VEGFR-1 was detected at 151-kDa (n=3).

CHAPTER 4. DISCUSSION

4.1 Overview

Newborns with CDH have abnormal diaphragm development, allowing the abdominal organs to herniate into the thoracic cavity and impair lung development. The main cause of death in CDH babies is occurred due to pulmonary hypertension. It results from the thickening of the medial and adventitial layers in the lung vessels leading to increased vessel resistance and ultimately heart failure. Despite the improvement in early diagnosis and postnatal management, the morbidity and mortality of CDH are high and the pathogenesis of pulmonary hypertension is poorly understood [2]. This poses a big challenge for pediatric surgeons, neonatologists and families. In response, Dr. Keijzer's group is dedicated to find the molecular mechanisms underlying pulmonary hypertension and pulmonary hypoplasia in CDH patients. We aim to develop a prenatal therapy to improve the outcome of newborns with this devastating congenital abnormality.

In our research group, we focus on the role of microRNAs (specifically miR-200b) in the pathogenesis of CDH and abnormal lung development. MicroRNAs are non-coding RNAs that regulate gene expression by inhibiting the translation of target mRNAs. Our lab previously discovered that the least hypoplastic lungs in the nitrofen rat model of CDH (the ones without a CDH) show an increased expression of miR-200b just before birth compared with normal lungs. Expression of miR-200b was lower at the early stages of abnormal lung development in the nitrofen lungs compared to control lungs. Prenatal administration of miR-200b mimics improved fetal lung development in the nitrofen CDH rat model [105]. To further investigate the role of miR-200b in lung development, we created a miR-200b KO mouse model. These mice exhibited

abnormal lung development due to increased fibroblast-like cells, dysfunctional surfactant and denser parenchyma. Our findings indicated that the development of pulmonary hypoplasia in CDH is associated with downregulation of miR-200b in the animal model of CDH and human patients [18]. Here, we investigated whether miR-200b has a role in abnormal pulmonary vessel development and pulmonary hypertension. To obtain a better insight into the role of miR-200b in pulmonary hypertension, we estimated changes in the right ventricle size and pulmonary arterial pressure using echocardiography in miR-200b KO mice. Interestingly, these mice exhibited increased pulmonary arterial pressure and cardiac enlargement (data are not published). For my MSc project, I evaluated the role of miR-200b in vascular remodeling associated with pulmonary hypertension in the miR-200b KO mouse model.

4.2 Vascular remodeling in miR-200b KO mice

In pulmonary hypertension, hypoxia changes the arrangement of the actin cytoskeleton in the pulmonary arterial smooth muscle cells, leading to an increase in α -actin and γ -actin fibers and ultimately excessive muscularization of the vessels. I used VVG staining to evaluate morphological changes of the pulmonary vasculature in adult miR-200b KO mice. Results showed that miR-200b KO mice have a 35% thicker arterial wall in their lungs compared with the WT group. Furthermore, the medial and adventitial wall thickness were increased in the miR-200b KO group. Thickening of the adventitial and medial layer is considered as the most prominent structural change in pulmonary hypertension.

Several studies have shown changes in the architecture of pulmonary vessels during embryonic development and adult life in both CDH patients and animal models. Roublova *et al.* showed

vascular structural changes in the surgically induced rabbit model of CDH from the pseudoglandular stage to the alveolar stage of lung development. They reported in fetuses with diaphragmatic hernia that the medial and adventitial wall were thicker than the control group in all stages of lung development especially the saccular and alveolar stage [106]. In another study, exposing 8-week-old rats to hypoxia resulted in thickening of the adventitia and media in the pre-acinar vessels. These vascular changes were associated with increases in the production of peri-vascular cells and subsequently, higher abundance of alpha-smooth muscle actin. Interestingly, vascular remodeling in hypoxic rat lung was completely reversed after 6 weeks of recovery at a normal level of oxygen (760 mm Hg) [110]. Similar vascular changes have been reported in human CDH lungs. Newborns with CDH displayed a significant increase for morphometric values including adventitial, arterial and total wall thickness compared with the control group [111]. To have a better understanding of vascular remodeling, I categorized the vessels into different groups relative to external diameter. In miR-200b null mice, I observed changes in the medial wall thickness in midsize arteries with an external diameter of less than 40 μm . This is the size of muscular arterioles. However, the degree of adventitial wall thickness increased in small arterioles with an external diameter of less than 20 μm . Taira *et al.* reported that in CDH patients changes in the degree of adventitial and medial wall thickness occur in all sizes of arteries from more than 500 μm to less than 75 μm [112]. They also measured the medial and adventitial area and revealed these parameters were significantly higher in arteries of CDH patients [112]. More recent studies have shown measuring adventitia or medial area, particularly the adventitia might not be an accurate indicator to represent vascular remodeling since usually the adventitial area has variable shapes around the vessels. In addition, tracing of the adventitial boundaries is challenging and creates significant errors in the morphometric

analysis [113]. Taira *et al.* performed another study and observed an increase in medial and adventitial wall thickness in all vessel sizes when they compared newborn and stillborn CDH patients with sudden infant death syndrome patients as a control group [114]. However, Shehata *et al.* observed a significant increase in the adventitial and medial wall thickness only in vessels of CDH patients with an external diameter of less than 200 μm which is the size of arterioles in human [111]. We observed changes in the pulmonary vascular structure in miR-200b KO mice similar to the morphologic vascular abnormalities observed in the lungs of CDH patients. This could be explained by the absence of normal transition at birth when pulmonary vessels cannot remodel after birth and the adventitia and media remain thick. These changes lead to an increased vascular resistance especially in the intra-acinar arteries, higher pulmonary arterial pressure and ultimately pulmonary hypertension in adult mice.

4.3 Three-dimensional micro-CT analysis of pulmonary vasculature

Small animal models have been used vastly to improve our understanding about pathophysiology of cardio-pulmonary diseases like pulmonary hypertension, CDH and congenital heart disease [30] [107] [115]. These studies mostly depend on stereological or histologic techniques. However, these techniques cannot evaluate the vascular changes in the entire lung. For instance, lung sections only represent approximately 0.1% of all pulmonary vessels [116] [107] [117]. Other techniques like plastination or corrosion casting provide visualization of the lung vasculature. However, with these techniques, quantitative measurements require specific equipment like a scanning electron microscope, which is semiautomated and time-consuming. Also, based on the Jiřík *et al.* study, filling of the microvessels by corrosion casting can be

influenced by the pressure of injection [117]. Micro-CT scan with a contrast agent has benefits over traditional techniques and provides an opportunity to explore the complexity of the microvasculature with 3D images and automated quantification in different organs. 3D, high-resolution images are obtained by taking approximately 2300 two-dimensional images using X-ray from different angles around the samples.

In my thesis, reconstructed micro-CT images with high resolution (8 μm) were used to measure total lung volume, the ratio of vascular volume to total lung tissue volume and the ratio of vascular surface area to total vascular volume. The micro-CT data did not reveal any significant differences between the pulmonary vasculature of the miR-200b KO and WT mice. This could be explained by the challenges related to the structure of the lung, perfusion, and optimization of contrast agent in the mouse animal model.

Micro-CT scan of the lung is more challenging than in other organs since, the lung has a complex vascular branching pattern, fragile microcirculation and a large number of vessels [118]. Also, lung capillaries have a close connection with the network of airways thus, with excessive pressure of perfusion, contrast agent easily breaks down capillary beds and penetrates alveoli and lung parenchyma. In my project, I tried to develop and optimize a new technique to perfuse the lung vasculature using a contrast agent in mice. Some of the challenges that I faced were overcome during the development of the technique however, more investigation and experiments are required to further optimize the perfusion technique. Very recently the first paper has been published about optimization of contrast agent in rat [115]. However, to the best of my knowledge, an optimized and reproducible method for perfusion of lung microvessels in mice has not been reported yet. Small size of the vessels in mice makes the procedure more complicated and to date, only two studies demonstrated the full complexity of the vascular

network in the mouse lung at the microlevel. In the first study, the restricted resolution of the micro-CT system did not allow the visualization of the microvessels in the distal lung and the smallest measured diameter of vessels was 160 μm while in the present study the smallest measured diameter of vessels was 8 μm . Also, the non-consistent distribution of contrast agent resulted in inadequate vascular resolution [119]. In the second study, the pulmonary vascular branching was demonstrated in 2 weeks old, 4 weeks old and adult mice with 3D reconstructed images [107]. However, the sample size was not provided in the study and the perfusion procedure was not clearly described. In addition, in their study, the amount of contrast agent to fill pulmonary vessels was determined based on visual checking of the vessels which was not accurate [107].

Consistent perfusion of contrast agent is the most important factor to obtain high quality and reliable vascular images in micro-CT. This procedure is associated with different variables that I encountered during my experiments:

- 1) Perfusion technique: I positioned and stabilized the catheter in the right ventricle to perfuse the pulmonary circulation. Since the right ventricle has a thin wall, it is easy for the catheter to be accidentally placed into the left ventricle allowing for contrast agent to flow into the systemic circulation. This will result in an insufficient amount of contrast agent in the lung vessels. It explains the observed variability of contrast agent distribution in the pulmonary vessels between samples from the same group (Figure 11). To address this issue, cannulation of the pulmonary artery via the jugular vein could be helpful. It provides a pathway for contrast agent to directly perfuse into the lung vessels [115]. However, cannulation in mice with very small sized vessels requires specialized equipment like a high-resolution dissection microscope and microsurgical expertise.

- 2) Contrast agent pressure: small changes in the pressure of contrast agent can affect the accuracy of post-micro-CT quantification analysis. For instance, high pressure of contrast agent leads to inflation of proximal arteries beyond the physiological condition or filling of the pulmonary veins. Very recently, Deng *et al.* have reported that matching perfusion pressure of contrast agent with pulmonary arterial pressure is the most important factor to achieve success in micro-CT imaging. They measured right ventricular systolic pressure (RVSP) for each individual animal and based on RVSP, perfused contrast agent. It means due to the effects of vascular remodeling on vessel pressure, miR-200b KO mice or other animal models of pulmonary hypertension require more contrast agent pressure during perfusion to achieve the same quality of micro-CT images [115].
- 3) Vascular flushing: removing clots and blood from the vessels is essential for successful contrast perfusion. I used PBS at room temperature to flush blood from the vessels. A recently published protocol suggested that to have better blood perfusion, the flushing solution should be warmed up to 37°C. This protocol also recommended that the animal body temperature should be maintained at 37°C during the process [115]. This helps to achieve better flushing of the pulmonary vasculature and effective exsanguination.
- 4) Air bubbles: the presence of air bubbles can make perfusion incomplete and subsequently cause errors in data analysis. This can be prevented by using appropriate connectors and tubes like ones I used in my experiments (Figure 3). Also, newly developed data analyzing software (Amira) can eliminate the bubbles leading to accurate data analysis [107]. However, this software is very expensive and was not available for my experiments.

- 5) Lung inflation: inflation of the airways can help to create a more natural condition for the vessels in reconstructed micro-CT images as their position will be closer to their normal position in the body.

Despite all the advantages of the micro-CT scan imaging technique, this technique has some limitations and as discussed above, the optimal use of contrast agent is time-consuming and challenging especially in mice. Furthermore, the technique requires euthanizing of the animals and does not allow for additional studies. Recently, a new method has been developed to do noninvasive angiography without contrast agent. In this technique, data filters are used based on the density of tissues. Then, 3D reconstructed images of the pulmonary vasculature are created from a large number of data points of vessel diameters. The analyzed data from this technique is compared with pulmonary angiography using a contrast agent. The results show a correlation between measured vessel diameter derived from the angiography with and without contrast agent. This approach is still under development and needs more investigation [120].

4.4 Involvement of miR-200b in the VEGF signaling pathway

This is the first study on the role of miR-200b in pulmonary hypertension by targeting the VEGF signaling pathway. I investigated the expression of VEGF-A and its receptors VEGFR-1 and VEGFR-2 in the lung of miR-200b KO mice and WT. Our data revealed that both miR-200b KO and WT groups express VEGF-A. However, the expression of VEGF-A was down regulated in miR-200b KO mice. In adult WT mice VEGF-A was expressed in the epithelium of the bronchioles and vascular smooth muscle cells of the pulmonary arteries. Maharaj *et al.* reported expression of VEGF-A only in the alveoli and epithelium of small bronchioles and not in the

large bronchioles [121]. This could be explained by the accuracy of their technique since they used a combination of IHC and *in situ* hybridization. The expression of VEGF-A in adult mice indicates the importance of this factor in the maintenance and stability of the vasculature system [122] [123].

VEGFR-1 expression was upregulated in the airways, large and small bronchioles and pulmonary arteries in miR-200b KO mice. While IHC showed a dramatic increase in the expression of VEGFR-1 in miR-200b KO mice, western blot did not show any significant differences between miR-200b KO and WT groups. This could be explained by the variation between animal samples. For the next experiments, my suggestion is to increase the number of samples for both IHC and western blot to obtain more reliable data. Also, in the present study, I used whole lung tissue for western blot however only left lung was used for IHC. My suggestion for future experiments is to only use left lung for IHC and western blot to keep the consistency between experiments.

VEGFR-2 expression did not show a difference between miR-200b KO and WT mice. Similar results have been reported by other researchers where in adult rats VEGFR-2 expression showed minimal variations between control and experimental groups of COPD or asthma. It was concluded that VEGFR-2 is not an appropriate indicator to represent the progress of angiogenesis. It supports the idea that VEGFR-1, with a high affinity for binding to VEGF-A, has a more critical role in the VEGF signaling pathway [124] [125].

The interaction between miR-200b and VEGF signaling pathway have been shown in other studies. In lung adenocarcinoma cells derived from *Kras*/ *p53*-mutant mice, downregulation of VEGFR-1 by miR-200b leads to suppression of invasion and metastasis [104]. Knocking down

of miR-200b in the retina of diabetic rats enhances the expression of VEGF-A at both the mRNA and protein levels [103]. Also, Downregulation of miR-200b promotes wound healing angiogenesis through VEGFR-2 and GATA-2 [125].

All together, increased VEGFR-1 as an anti-angiogenic factor inhibits the normal angiogenic effects of VEGF-A. This might cause endothelial cell dysfunction and maybe the loss of proper crosstalk between endothelial cells and smooth muscle cells of arteries and ultimately vascular remodeling in miR-200b KO mice. The mechanism is not clear yet and needs more investigation.

4.5 Conclusion

Pulmonary hypertension in miR-200b KO mice is associated with changes in vascular morphology. Our results suggest that the absence of miR-200b leads to pulmonary hypertension by vascular remodeling due to increased thickness of the medial and adventitial layers of the pulmonary vessels, especially in the intra-acinar arteries. MiR-200b could also contribute to pulmonary hypertension by downregulation of VEGF-A and upregulation of VEGFR-1. We hope these findings will help to develop a future therapeutic approach to reduce the severity of pulmonary hypertension in CDH patients.

4.6 Future direction

In my project, I tried to optimize micro-CT scan in the lung of mice. As discussed above, I figured out some challenges related to the perfusion procedure. However, more experiments are needed to establish a reproducible quantification technique. After establishing a reliable protocol,

a combination of micro-CT data with histology assessment will provide a cohesive understanding of the changes occurring in the pulmonary vascular phenotype.

I used IHC to show the expression of VEGF-A and its receptors VEGFR-1 and R-2. I also performed western blotting only for VEGFR-1. So, for the next step, my recommendation is to assess the expression of VEGF-A and VEGFR-2 in miR-200b KO mice compared with WT using quantitative techniques like ELISA or western blotting to validate the IHC results. I also suggest having a closer look at the interaction between miR-200b and the VEGF signaling pathway by assessing the expression of mediators or downstream components in the VEGF signaling pathway. For instance, evaluating the expression of phosphoinositide-3-kinase (PI3K) as an important downstream factor of VEGF signaling in angiogenesis.

CHAPTER 5. REFERENCES

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