Identification of the organ-specific function of lymphatics focusing on the neonatal lung

Ph.D. thesis

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1. Introduction

In addition to the classical functions of lymphatic system, it has become clear that lymphatics has organ specific functions as well. Our aim was to identify these functions of lymphatics, focusing on the neonatal lung and adult skin.

At birth, to establish respiration, lung development requires a complex prenatal pulmonary maturation program. Surfactant plays a critical role in this process and is central to the treatment of respiratory disorders. However, current therapies have limitations, necessitating the development of new methods. It is well-documented that late gestation embryo performs fetal breathing movements (FBMs), but the physiological importance of these events is not clear. This led us to define the physiological role of FBMs in preparation for the first breath.

Prior experimental approaches for studying FBMs had significant limitations. *Clp1*$^{K/K}$ kinase-dead mice lose the innervation of skeletal muscles from E16.5 onward, resulting in impaired motor function and respiratory failure. Importantly, Hanada et al (2013) described normal lung development. That is why we used Clp1 mice strain to test the possible role of FBMs.

To investigate organ specific lymphatic function, we aimed to induce the lymphatic growth in the skin with nucleoside-modified VEGFC mRNA-LNP approach.

2. Objectives

We have had two aims. First was to define and to characterize the role of FBMs in the neonatal lung, second was to develop a new approach to study the role of lymphatics in adult skin.

I. To define the physiological role of FBMs in preparation for air inflation of the prenatal lung at birth, we used the *Clp1*$^{K/K}$ mouse strain as a genetic model which has denervation of skeletal muscles during late gestation.
   1. Our aim was to characterize the phenotype of *Clp1*$^{K/K}$ mouse strain.
   2. Next, we aimed to describe lung development in *Clp1*$^{K/K}$ embryos compared to control.
   3. We also wanted to characterize the lymphatic function in late gestation embryos lacking FBMs.

II. Our second big goal was to investigate lymphatic growth in the skin. For that our aim was to develop a system to induce organ-specific lymphatic growth in the skin. One of the most important growth factors of lymphatics is VEGFC. mRNA-LNP platform is an effective new therapeutic tool for protein production. So, our aim was to investigate and characterize whether nucleoside-modified VEGFC mRNA-LNPs could effectively induce the growth of new lymphatic vessels in the skin of adult mice.

3. Methods

3.1. Animals

For neonatal lung experiments mice carrying the kinase-dead Clp1 allele (*Clp1*^{K}) were maintained on c57Bl/6 genetic background. For monitoring lymphatic function in the lung, *Flt4*^{YFP} mice were crossed to kinase-dead Clp1 mouse strain and maintained in heterozygous form on the c57Bl/6 background.

For experiments investigating lymphatic vessels in the skin, we used 6–12-week-old *Prox1*^{GFP} lymphatic reporter animals which were obtained from the Mutant Mouse Regional Resource Centers and were maintained in heterozygous form.

3.2. Timed Matings and Handling of Late Gestation Embryos and Newborns

Clp1$^{K/+}$ heterozygous animals were used to set up overnight timed matings. Embryos and newborns were collected at E14.5, E15.5, E16.5, E17.5, E18.5, E19.5 and P0. The whole chest, isolated lungs, gut and skin were used for histology, weight and DNA content measurements.

3.3 Histological Processes and Immunohistochemistry for Neonatal Lung Experiments

Embryonic and newborn tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight on 4°C, dehydrated then embedded in paraffin using a Leica EG1150H embedding station. Tissue sections were generated using an

HM340E Thermo Scientific microtome and processed for hematoxylin-eosin (HE) (Leica), periodic acid-Schiff (PAS) (Sigma-Aldrich), trichrome (Sigma-Aldrich) and immunohistochemistry staining. Microscopic images were taken by a Nikon ECLIPSE Ni-U microscope connected to a Nikon DS-Ri2 camera.

3.4 DNA Content Measurements

For total DNA content measurements DNA was isolated from whole lungs using DNeasy blood and tissue isolation kit (Qiagen). DNA concentration of the whole lungs was measured by NanoDrop OneC Microvolume UV–Vis Spectrophotometer (Thermo Scientific).

3.5 Monitoring Pulmonary Lymphatic Function *in vivo*

To monitor pulmonary lymphatic function in the developing lung we crossed the CLP1 strain to lymphatic reporter ($Flt4^{YFP}$) background. Pregnant females were anesthetized. Large molecular weight, 70 kDa fluorescent labeled macromolecule rhodamie-dextrane (RhD) was injected into the developing lung of these mouse embryos at E18.5.

3.6 Design, production of VEGFC mRNAs and LNP formulation of Poly(C) and VEGFC mRNAs

Norbert Pardi from University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, USA designed and

produced the nucleoside-modified mRNAs. Barbara L. Mui, Ying K. Tam, Thomas D. Madden and Michael J. Hope from Acuitas Therapeutics, Vancouver, BC, Canada developed and prepared the lipid nanoparticles.

3.7 Monitoring in vivo lymphatic growth in the skin of adult mice

6–12-week-old $Prox1^{GFP}$ lymphatic reporter mice were intradermally injected with 1 μg of Poly(C) (Control) or VEGFC mRNA-LNPs into the back skin. Lymphatic growth in lymphatic reporter animals was visualized by fluorescent stereo microscopy using a Nikon SMZ25 microscope connected to a Nikon DS-Ri2 camera. For paraffin-based histology we used the exact same method as described in the neonatal lung experiments in chapter 3.3. Skin sections were processed for immunohistochemistry staining. Microscopic images were taken by a Nikon ECLIPSE Ni-U microscope connected to a Nikon DS-Ri2 camera.

3.8 Presentation of Data and Statistical Analysis

NIS-Elements Imaging (Nikon), Fiji Software (NIH), and Adobe Photoshop were used for image processing and analysis. For statistical analysis GraphPad Prism 7.0 and Microsoft Office Excel software programs were used.

4. Results

4.1 *Clp1*$^{K/K}$ Embryos and Newborns Exhibit Impaired Skeletal Muscle Function Including FBMs

At first, we set up timed matings with *Clp1*$^{K/+}$ mice to characterize the phenotype of *Clp1*$^{K/K}$ and control (*Clp1*$^{+/+}$) embryos and newborns. Newborn mice carrying two kinase dead CLP1 alleles (*Clp1*$^{K/K}$) showed impaired respiration and motility, or a complete lack of breathing and movement. *Clp1*$^{K/K}$ newborns were cyanotic and presented signs of acute respiratory failure and died shortly after birth. While control *Clp1*$^{+/+}$ mice exhibited normal breathing, movement and color. The weight and size of *Clp1*$^{K/K}$ and *Clp1*$^{+/+}$ control mice showed no difference. Lungs of *Clp1*$^{K/K}$ newborns show reduced alveolar area and thickened alveolar septa compared to the *Clp1*$^{+/+}$ littermate controls.

4.2 Late Gestation *Clp1*$^{K/K}$ Embryos Do Not Show Altered Expression of Molecular and Cellular Markers of Lung Development

Next, we characterized late lung developmental markers in *Clp1*$^{K/K}$ and *Clp1*$^{+/+}$ mice before birth. The expression levels of these markers were normal; including CC10 (Club cell 10), alveolar type II cells (SP-C-surfactant protein c), type I cells (PDPN-podoplanin), mesenchyme (PDGFRα, Vimentin, Desmin), vascular smooth muscle cells and pericytes (PDGFRβ,

NG2, and α-SMA), lung endothelial cells (PECAM1). Markers of pulmonary LECs: VEGFR3, PROX1, and LYVE1 showed normal expression levels with no major difference in the number of cells or vascular structures in *Clp1*$^{K/K}$ compared to control *Clp1*$^{+/+}$ mice.

4.3 Late Gestation Lungs of *Clp1*$^{K/K}$ Embryos Show Thickened Alveolar Septum and Reduced Alveolar Area Before Air Inflation

Then we investigated lung morphology of *Clp1*$^{K/K}$ and *Clp1*$^{+/+}$ embryos in each embryonic day from E14.5 to birth. HE stained sections revealed no detectable changes before E16.5 in *Clp1*$^{K/K}$ embryos in the examined parameters. However, at E17.5 we detected thickened alveolar septum and reduced alveolar area in *Clp1*$^{K/K}$ embryos compared to control *Clp1*$^{+/+}$ embryos. These changes were detectable at E18.5 and E19.5 as well. To further support normal lung development, we performed PAS and trichrome staining. We could not detect any difference between *Clp1*$^{K/K}$ and the control late gestation lungs with Trichrome staining, which would indicate fibrosis. In addition, PAS staining showed normal levels of glycogen in the alveolar cells of *Clp1*$^{K/K}$ embryonic lungs at E18.5. DNA content and dry weight of *Clp1*$^{K/K}$ embryonic lungs were also normal at E18.5.

4.4 The Pulmonary Lymphatic Vessels Are Markedly Dilated in Late Gestation *Clp1*$^{K/K}$ Embryos Before Air Inflation

To characterize growth of pulmonary lymphatic vessels in *Clp1*$^{K/K}$ embryos we investigated embryos at different time points from E14.5 to E19.5 and performed immunostaining with lymphatic markers. Lymphatic vessels appeared at E14.5 and developed normally in the *Clp1*$^{K/K}$ embryos as well as in control *Clp1*$^{+/+}$ embryos. Importantly at late gestation, at E17.5, E18.5, E19.5 lymphatic vessels appeared to be dilated in the *Clp1*$^{K/K}$ mice compared to the *Clp1*$^{+/+}$ mice. However, in other organs such as small intestine and skin, the structure of lymphatic vessels showed no difference at E17.5, E18.5, E19.5 in the *Clp1*$^{K/K}$ compared to the *Clp1*$^{+/+}$ control embryos.

4.5 Lymphatic Function Impairment Shown in Late Gestation Lungs of *Clp1*$^{K/K}$ Embryos

To monitor lymphatic function, we crossed Clp1 mice with lymphatic reporter mice (*Flt4*^{YFP}). Large molecular weight (70 kDa) fluorescent labeled macromolecule (RhD) was injected into the developing lung of these mouse embryos. We detected decreased RhD signal in the lymphatic vessels of late gestation (E18.5) *Clp1*$^{K/K}$ embryos compared to the controls. These results indicate that lymphatic function was severely reduced in *Clp1*$^{K/K}$ embryos at E18.5 compared to littermate controls.

4.6 Local lymphatic growth in vivo by administration of VEGFC mRNA-LNPs into back skin

To investigate organ-specific lymphatic growth in the skin we used *Prox1*^{GFP} lymphatic reporter mice, in which lymphatic endothelial cells express GFP (100). Nucleoside-modified VEGFC mRNA-LNPs were injected intradermally into the back skin of *Prox1*^{GFP} lymphatic reporter mice. Major increase in lymphatic growth was detected after 22 days which is shown by fluorescent stereo microscopy by detecting the Prox1-GFP signal. It is also detected by LYVE1 expression of lymphatic endothelial cells in paraffin-based histology slides.

5. Conclusions

Surfactants have been considered the most important factors in lung development to prepare the lung for air inflation at birth. A prior study of our research group has reported that lymphatic function is a previously unknown factor which increases lung compliance before birth. Therefore, lymphatics have an important role in preparation for the inflation at birth. We aimed to better understand what other factors and mechanisms could play role in late gestation and at birth. We could confirm that late gestation embryos of *Clp1*$^{K/K}$ mice exhibit a progressive loss of motor neurons and skeletal muscle activity from E16.5 onward during late gestation and are lacking fetal breathing movements. We think this model is excellent for studying the impact of FBMs on lung development and gives another view compared to previous other approaches like leakage or drainage of the amniotic fluid, performing heroic surgery, or paralysis starting at an early developmental stage, etc., which all have great limitations.

Our next aim was to describe cellular and molecular lung development of *Clp1*$^{K/K}$ late gestation embryos lacking FBMs. We could conclude that it was normal, and no significant difference was detectable between *Clp1*$^{K/K}$ and control *Clp1*$^{+/+}$mice. However, our results demonstrated impaired lung

expansion represented in thicker alveolar septa, reduced alveolar area in *Clp1*$^{K/K}$ late gestation embryos which indicates that mechanical forces including FBMs may affect the expansion of the developing lung.

Our following aim was to characterize the lymphatic function in late gestation embryos lacking FBMs. Pulmonary lymphatics are developing and are present in the lungs of *Clp1*$^{K/K}$ embryos, but they are markedly dilated at late gestation. Importantly, late gestation *Clp1*$^{K/K}$ embryos show reduced prenatal lymphatic function as well. Therefore, our results indicate that lymphatics play an important role in lung development for the preparation to the first breath.

Another aspect which could provide valuable information in the future is to stimulate FBMs during late gestation to study whether it could be an effective way to reduce the risk of the development of neonatal respiratory failure.

Our final objective was to characterize a system to induce organ specific lymphatic growth in the skin. With nucleoside-modified VEGFC mRNA-LNP platform we could effectively increase local lymphatic growth in the back skin dose dependently. In the future it may open new aspects of the treatment of lymphedemas.

New results in points:

- Our results show impaired lung expansion represented as the alveolar septa are thicker, and the alveolar area is reduced in *Clp1*$^{K/K}$ late gestation embryos.

- Our findings suggest that mechanical forces including FBMs influence the expansion of the developing lung, while the expression of molecular markers of lung development are not affected. We could not detect lung hypoplasia.

- *Clp1*$^{K/K}$ late gestation embryos have dilated pulmonary lymphatic vessels, display reduced prenatal lymphatic function and impaired lung expansion. Our results have revealed the previously unrecognized role of FBMs in prenatal lung expansion, suggesting that FBMs and prenatal pulmonary lymphatics function together to prepare the developing lung for inflation and gas exchange at birth.

- We developed a system to induce organ-specific lymphatic growth in the skin. Nucleoside-modified VEGFC mRNA-LNPs effectively increased local lymphatic growth in back skin.

6. Bibliography of the candidate's publications

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