MARSHALL PLAN SCHOLARSHIP
FINAL REPORT

THE ROLE OF TCF21 IN POSTNATAL SURFACTANT BIOSYNTHESIS

by

Veronika SCHWABL

Internal Supervisor: Christoph Wiesner, Dr

External Supervisor: Michelle Tallquist, PhD

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Declaration of honour

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Abstract

The Transcription factor Tcf21 plays a crucial role during embryonic morphogenesis of several organs. It is also essential during the differentiation process of alveolar Lipofibroblasts during embryonic lung development.[1] Using an inducible Cre-loxP mouse model, transgenic mice missing Tcf21 were generated and analyzed using qRT-PCR and several histochemistry methods. We observed that the Tcf21 gene regulates lipid metabolism gene expression in the postnatal lung since the deletion of Tcf21 resulted in decreased levels of lipid-metabolism gene expression and a decreased neutral lipid content in Tcf21 knockout mice. Furthermore, the deletion of Tcf21 resulted in a decreased population of alveolar epithelial cells as well as a reduced expression of surfactant proteins. Hence one can conclude that Tcf21 expressing lipofibroblasts control alveolar cell differentiation and decreased lipid contents in the lung may affect surfactant biosynthesis due to impaired ATII differentiation.

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1 Introduction

1.1 Development of the Pulmonary System

The developmental processes of the pulmonary system are induced and tightly controlled by several hormonal pathways.[2, 3] In most air-breathing species the respiratory system develops not during gestation but its development and maturation continue also postnatally.[2-5] In human, this implies completion of development only around the age of 36 months.[4, 6] However, the development of the respiratory system has its peak in the third trimester of human pregnancy. Approximately at week 36 of gestation, the human lung is mature, facilitating sufficient gas exchange after term birth, even before the lung is fully developed.[5]

When monitoring the histological development of lung tissue during the fetal period, which is following the embryonic period (ending around the 8th week of pregnancy in humans), four different stages, called the pseudoglandular, canalicular, terminal saccular and alveolar stage, become obvious.[3] During the first three stages of lung development, the conducting airways, the bronchi, and the bronchioles form and alveolar epithelial cell types start to differentiate.[3, 6, 7] The last stage of pulmonary development, starting a few weeks before birth and continuing until early childhood, is the alveolar stage. During this time, most of the blood-air-barrier is formed by secondary septation, where a secondary septum evolves out of the primary septum dividing preexisting saccules into smaller alveoli and thereby increasing the surface of the respiratory system significantly. The process of alveolarization is crucial for a fully functioning lung and can be affected by many physical factors.[3, 8]

1.2 Alveoli and their epithelium

In the lung, alveoli form the blood-air barrier where the gas exchange takes place. The trachea gives rise to bronchi which in their turn segue into bronchioles, connecting alveoli which are joined together into alveolar saccules. This structure is

evoked by a process occurring in many different organs during embryonic development called branching morphogenesis, in which a tubular network is formed out of epithelial and endothelial cells.[9]

The alveolar epithelium is lined up by two different cell types: while the thin, flat alveolar type 1 (AT1) cells are facilitating the gas exchange at the blood-air barrier, AT2 cells are mainly responsible for producing and secreting surfactant into the alveoli, assuring its stability and preventing atelectasis.[6, 10, 11]

In humans, alveoli have a diameter of approximately 0.2 mm and their epithelial wall shows a depth of 0.2 µm. Forming the blood-air barrier, alveolar epithelial cells are surrounded not only by extracellular matrix (ECM) but also by a dense capillary network. Due to their oval shape, low diameter and watery mucus coat, alveoli would collapse and stick together after total exhalation. This would cause severe damage to epithelial cells and therefore impair gas exchange forever. Hence, alveolar epithelial cells secrete surfactant, a heterogeneous surface active agent, that prevents atelectasis by reducing surface tension but increasing surface pressure.[6]

Not only surfactant biosynthesis but also the reuptake of all surfactant components and recycling them is partially done by AT2 cells.[11]

1.3 Pulmonary Surfactant

Surfactant (SURFace ACTive AgeNT) consists of proteins and peptides but by weight, 90% of surfactant are lipids including neutral lipids such as cholesterol, but mostly saturated phospholipids like phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, sphingomyelin or phosphatidylcholine (PC), to be more specific either lyso-PC containing one palmitic acid side chain or Dipalmitoyl-PC (DPPC) with 2 palmitic acid side chains, being the molecule in surfactant that is the most responsible for the mixture's surface-active character. [6, 10-12] Also several types of unsaturated PC molecules are components of surfactant. [11]

The surfactant in the alveoli is not only a thin layer of active molecules but rather a multilayer coat with a mesh-like structure, called tubular myelin, caused by interactions between lipids and surfactant proteins. Since the alveolar size is not directly constant, changes of surface area are common, the top layer of surfactant can form bubbles which are then transported to alveolar epithelial cells or alveolar macrophages where the surfactant components are either recycled or digested.[6, 12]

To ensure all these mechanisms of pulmonary surfactant, enough surfactant must be present. Healthy term infants showed an overall amount of 100mg surfactant per kg bodyweight. (In this context, the overall amount of surfactant refers not only to the active surface layer but includes the amounts of intra and extracellular surfactant as well.) In contrast, surfactant-deficient preterm infants exhibit a tenth of this amount only. To compensate for this dramatic deficiency, artificial surfactant is provided.[6]

Four particular proteins have been identified to be part of surfactant, namely SP-A, SP-B, SP-C, and SP-D. These four proteins make up approximately 10% by weight. The larger and more frequent surfactant proteins SP-A and SP-D consist of distinct subunits, are hydrophilic and support the immune system in eliminating foreign particles and pathogenic organisms inhaled into the alveoli. Whereas SP-B and SP-C are smaller, hydrophobic and their interaction with phospholipids, affixing them at the air-liquid interface, causes the reduction of surface tension.[6, 10, 11]

1.4 Interactions between pulmonary Lipofibroblasts and AT2 cells

Pulmonary Lipofibroblasts are fibroblasts that express the glycoprotein Thy-1 and contain characteristic lipid vesicles. This cell population benefits from the stored lipid droplets since triglyceride protect them from oxidative injuries.[13] Lipofibroblasts share some characteristics with adipocytes but also other mesenchymal cells such as smooth muscle cells and pericytes.

In the lung, the main role of lipofibroblasts is thought to be providing AT2 cells with triglycerides for surfactant synthesis. Studies showed that in contrast to AT2 cells,

lipofibroblasts are able to uptake and store triglycerides. Furthermore, it was observed that the triglycerides uptaken by lipofibroblasts are converted by AT2 cells into phospholipids, which are an important constituent of the pulmonary surfactant. With increased uptake of lipofibroblast-provided triglycerides, AT2 cells also increase their uptake of extracellular glucose since both molecules are necessary for the formation of phospholipids such as desaturated phosphatidylcholine or phosphatidylglycerol.[12, 13]

The main problem in chronic lung diseases in premature births is not only the prevalent surfactant deficiency, which could be easily remedied by replacing it with artificially produced surfactant but more severe is the missing communication and homeostasis between AT2 cells and lipofibroblasts. Currently, neonatology provides premature births with continuous positive airway pressure (CPAP) to maintain just the right amount of physical stretch for alveolar expansion so PGE2 and PTHrP signaling between AT2 cells and lipofibroblasts are promoted.[13] However, this procedure comes with its drawbacks since too high pressure can lead to injuries of AT2 cells or lipofibroblasts resulting in a surplus of lipofibroblasts transdifferentiating into myofibroblasts since alveolar overexpansion results in a decrease of AT2 cell-secreted PTHrP causing the overstimulated trans-differentiation. Lack of PTHrP-receptor expressing lipofibroblasts consequences in decreased surfactant production, starting a vicious circle of formation of incompetent alveoli, the main characteristic of bronchopulmonary dysplasia (BPD).[13]

1.5 Role of Tcf21

Transcription factor 21 (Tcf21) is also known as Pod1, capsulin or epicardin and is a class II basic helix-loop-helix (bHLH) transcription factor encoded by its respective gene TCF21. Tcf21 has a crucial role during the embryonic morphogenesis of several organs (for instance the respiratory, cardiovascular or urinogenital system). This protein is strongly expressed in the respiratory system, kidneys, and placenta, as well as in the embryonic epicardium and tissues that are derived from mesenchyme, such as guts and gonads. [14-17].

An antisense long non-coding RNA called TARID (which means TCF21 Antisense RNA Inducing Demethylation) is in charge of regulating the activation of transcription factor TCF21, as it persuades the promotor's demethylation. By interoperating with several transcriptional regulators, TARID influences the gene expression level as well.[18] TARID itself is activated by several pathways, that are triggered by ligand-binding to a PDGF-β receptor, a cell surface tyrosine kinase receptor, which induces phosphorylation in order to induce signal transduction.[19] TCF21 is significantly essential during embryogenesis of the lung and other tissues. Several studies showed that mice lacking TCF21died shortly after being born as they were not able to develop functioning alveoli nor glomeruli.[20] Furthermore, the development of cardiac fibroblast could not take place in these mice. Instead of turning into fibroblasts, the cells on the heart surface evolved into smooth muscle cells more frequently.[17] Another important function of transcription factor TCF21 is its role as a tumor suppressor.[21]

Furthermore, Tcf21 can be used to identify pulmonary lipofibroblasts even in the absence of the characteristic neutral lipid droplets. Thus, Tcf21 can be potentially associated with lipid storage regulations. [1]

1.6 Aim of the Thesis

The aim of this thesis was to identify the role of Tcf21 in the lipid metabolism of pulmonary lipofibroblasts and how its absence is affecting the proliferation of lipofibroblasts and continual Alveolar Type 2 (AT2) cells as well as the production of surfactant.

In healthy lungs, the lipofibroblast's duty is to process neutral lipid droplets for AT2 cell uptake. As shown by our lab, Tcf21 is essential for the differentiation process of alveolar lipofibroblasts during embryonic lung development.[1] Tcf21 knockout mice showed a lower number of lipofibroblasts and AT2 cells, suggesting that the loss of Tcf21 effects the lipid metabolism of lipofibroblasts which in turn is crucial for successful lipofibroblast-AT2 cell interactions. Restrictions in lipofibroblastic lipid handling hence would result in decreased surfactant synthesis as AT2 cells depend on

neutral lipids provided by lipofibroblasts during surfactant biosynthesis. Therefore, we assume that the loss of Tcf21 results in decreased amounts of surfactant.

Using an inducible Tcf21 Cre-loxP mouse model transgenic mice were generated. After induction on postnatal day 2, activated nuclear Cre deletes Tcf21 in lipofibroblasts. The mice are then sacrificed at different time points to obtain data allowing comparisons between different stages of pulmonary development. Lung samples were prepared for histochemistry and qRT-PCR. Various methods are used to investigate the amount and distribution of neutral lipids in the perinatal lungs, the expression levels of several lipid metabolism associated genes and proteins as well as alveolar epithelial cell markers.

2 Materials and Methods

2.1 Mice

Tcf21$^{mCrem/+}$ [22] (Jackson Labs, 007669), Tcf21^{fl} (kindly provided by Dr. T. Quertermous at Stanford University), and *R26R^{tdT}* (Jackson Labs, 007914)[23], were maintained on a mixed C57Bl/6 background. Tcf21^{mCrem}; *R26R$^{tdT/tdT}$* mice were backcrossed a minimum of four generations to C57Bl/6 and contained the J mutation of the NNT gene. Here and following the term “mutant” refers to the genotype Tcf21$^{mCrem/fl}$, whereas “control” mice either show a Tcf21$^{mCrem/+}$, Tcf21$^{fl/+}$ or Tcf21$^{+/+}$ genotype. Induction of the hormone tamoxifen causes the cytoplasmic estrogen receptor-bound inducible Cre recombinase (mer-Cre-mer) to translocate into the nucleus where it spatiotemporally recombines loxP sites, thus regulates gene expression by knocking out the target gene Tcf21. The Tcf21 lineage tracing, Tcf21$^{mCrem/+}$; *R26R$^{tdT/tdT}$* and a conditional knock-out mouse, Tcf21$^{mCrem/fl}$; *R26R$^{tdT/tdT}$*, was generated by selective breeding of Tcf21$^{mCrem/+}$ and loxP lines. RTT (Rosa26 Tandem-dimer Tomato) was used as reporter gene. Male and female mice were used for all following procedures. Mice were housed in a temperature- and humidity- controlled facility with a 12-h light/dark cycle. All procedures were approved by the University of Hawaii Institutional Animal Care and Use Committees and were conducted in accordance with the NIH guidelines for care and use of laboratory animals.[1, 24]

2.2 Tamoxifen Induction

Tamoxifen (MP Biomedicals; 0215673891 or Fisher Scientific, AdipoGen; 50-149-0595) was dissolved in a 90% sunflower seed oil solution containing 10% ethanol to obtain a 20 mg/mL stock solution. For postnatal induction, the tamoxifen containing solution was diluted in the sunflower seed oil to a concentration of 5 mg/ml. Mice were induced at postnatal day 2 (p2) at a dose of 0.15 mg/g body weight by a single intragastric injection. Without the administration of tamoxifen, no reporter activity was observed at any time (preliminary data, not shown in this work).

2.3 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

For this procedure whole lungs, right or left lung lobes from mutant and control mice of all genotypes were used. Isolated lung tissues were lysed in TRIzol® Reagent (Thermo Fisher Scientific, 15596026). Total RNA was prepared according to the manufacturer's recommendation. RNA concentration and quality were determined by NanoDrop ND-1000 (Thermo Fisher Scientific) and 2 % agarose gel electrophoresis. For first strand cDNA synthesis from total lung RNA, M-MLV Reverse Transcriptase and buffer (Sigma, M1302-40KU) and Random Hexamer Primers (Thermo Fisher Scientific, SO142) were used. RNA from input and IP samples was reverse transcribed using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, 11766050). RT-qPCR reactions were performed with PowerUp™ SYBR™ Master Mix (Fisher Scientific, A25776) on the LightCycler® 480 II instrument (Roche, Serial# 6352), using the Multiwell Plate 384. Reactions were performed in a total volume of 5 µL with the following cycling conditions: 50 °C 2 min, 95 °C 10 min, 50 cycles of 95 °C 15 s, 58 °C 45 s and 72 °C 1 min, followed by one cycle melting curve. Fold-enrichment was calculated using the ΔΔCt method, normalized to GAPDH expression. Housekeeping gene quantity was consistent across samples. ΔCt was determined by subtracting the Ct of the reference gene from that of the target gene. Relative starting quantity was calculated based on Ct-values of Standard Curve samples for each series of experiments. Primer sequences available upon request.[1, 24]

2.4 Tissue processing and staining procedures

Perinatal lungs were isolated at selected time points and fixed with freshly prepared either 1% paraformaldehyde (PFA) in DPBS overnight at 4 °C. Tissues were sequentially incubated with 30 % sucrose in DPBS for 4 hrs, and 1:1 mixture of 30 % sucrose in DPBS and Tissue-Tek® optimal cutting temperature (O.C.T)TM (VWR, 102094-106) for 1 h. Tissues were then embedded in Tissue-Tek® O.C.TTM medium and flash frozen with dry-ice cold 2-methyl butane (Fisher Scientific, O3551-

4). From frozen embedded lungs 10µm sections were sliced and placed onto charged slides and stored at -20°C for further staining procedures. Prepared tissue sections were used hematoxylin and eosin (H&E) staining as well as Oil Red O (ORO) staining.

2.4.1 H&E staining

Haematoxylin and eosin (H&E) staining was performed to examine tissue morphology, according to standard procedures on hydrated 10 µm sections, placed in Working Mayer's Hematoxylin Stain (Cat#H08-20R) for 5 min. Sections then were first rinsed with tap water for ≥ 5 min and deionized water for 1 min, followed by 95 % EtOH solution for 1 min. Sections were counterstained in eosin Y Stain (Cat#07-20R) for 30 s, dehydrated in first 95 % EtOH and second anhydrous alcohol, cleared in two changes of Xylene solution for ≥ 2 min and finally being mounted using Omnimount® (National diagnostics, Lot#03-10-26) with a 55 mm glass coverslip. Haematoxylin and eosin stained sections were imaged by a Zeiss Axioskop 2 Plus light microscope equipped with a digital camera (AxioCamMR3), using AxioVision v4.7.2.0 software. All images were processed (sizing, brightness or contrast adjustments) using Fiji ImageJ (v2.0.0).

2.4.2 ORO staining

To determine the amount of neutral lipids in the lung tissue, 10µm sections were stained using Oil Red O solution. Therefore, Oil Red O powder was dissolved in 60% isopropanol and used immediately. Prior to staining, the 0.6% ORO staining solution was filtered. The frozen tissue sections were stained for 15 min at RT. Tissue sections were washed with running tap water for 20 min and then mounted with 66% glycerol under glass coverslips. sections were imaged by a Zeiss Axioskop 2 Plus light microscope equipped with a digital camera (AxioCamMR3), using AxioVision v4.7.2.0 software. All images were processed (sizing, brightness or contrast adjustments) using Fiji ImageJ (v2.0.0). Tissue area and area of lipid droplets per field of view was quantified using ImageJ by changing images to 8-bit and adjusting

the threshold of each image to reduce background. Percental tissue area was calculated by analyzing particles (size: 0 – Infinity pixels2, circularity: 0.25 – 1, show: outlines).

3 Results

3.1 Lung organogenesis depends on Tcf21 lineage

Our recent data showed, that Tcf21 expressing cells in the lung are primarily lipofibroblasts and play an important role during alveolarization and maturation of the pulmonary system.[1] Hence, the data shown now focused on the role of Tcf21 during the postnatal surfactant synthesis and its effect on the lipid metabolism and communication between alveolar type 2 epithelial cells and pulmonary lipofibroblasts. Therefore, the expression of lipid metabolism genes, surfactant protein genes and cell type-specific genes at different time points were elucidated as well as the amount of neutral lipids in the postnatal lung determined and compared.

3.2 Tcf21 deletion is most severe with euthanization at earlier timepoints

To investigate the importance of Tcf21 in the organism, we determined the effect loss of Tcf21 has on the lung. Using an inducible Cre-lox mouse model we were able to delete Tcf21 postnatally. Most data presented in this thesis were obtained conducting the respective experiments at three different time points. The deletion of Tcf21 was induced at postnatal day 2 but samples were collected at the age of one, two or four weeks (p7, p14 or p28). To determine the deletion efficiency of Tcf21 for all time points qRT-PCR was conducted (Figure 1).

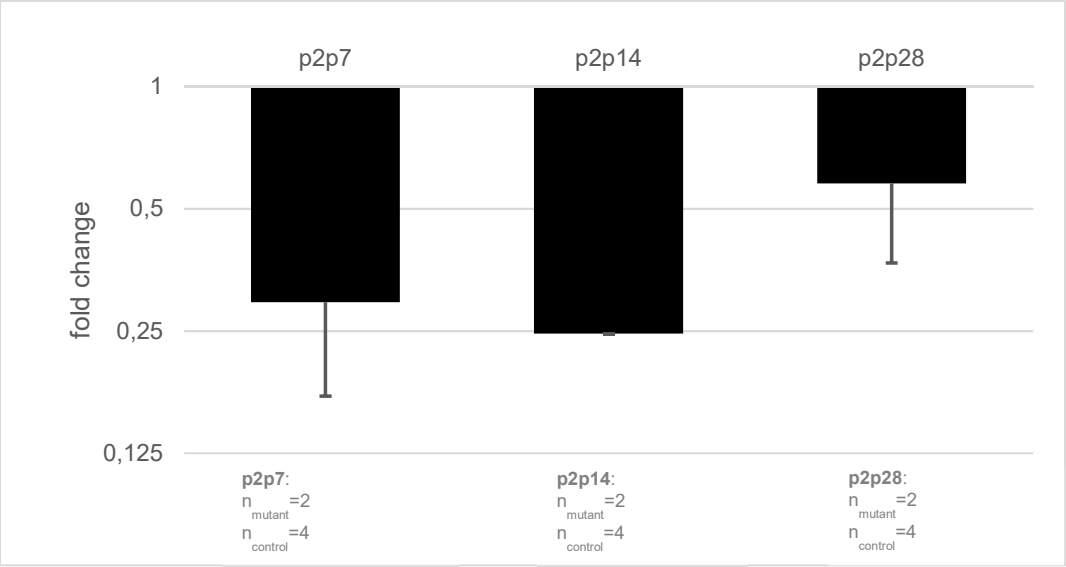


Figure 6: Tcf21 deletion effect at different time points. *Tcf21 deletion in mutant mice due to tamoxifen induction at postnatal day 2 (p2). Samples were either collected at p7, p14 or p28. After lung RNA isolation and qRT-PCR, the expression of Tcf21 in mutant mice was compared to the Tcf21 level in control mice of the same age. Samples were normalized by GAPDH expression level. qRT-PCR was performed in technical triplicates. Standard deviation is shown with error bars. Tcf21 deletion works at all time points.*

Deletion of Tcf21 using 0.15mg Tamoxifen/g bodyweight was effective for all time points (Figure 1). Compared to control mice, mutant mice showed lower expression levels of Tcf21 in the whole lung. Especially samples that were collected at earlier time points (p7 and p14) showed a more severe Tcf21 deletion compared to samples collected at p28. However, no time point showed a perfect deletion of 100 percent.

3.3 Lipid metabolism gene expression is decreased in Tcf21 knockout lungs

By intra gastric tamoxifen induction, Tcf21 was deleted in mutant mice to determine the effect of postnatal Tcf21 loss on the expression of various lipid metabolism genes (Figure 2).

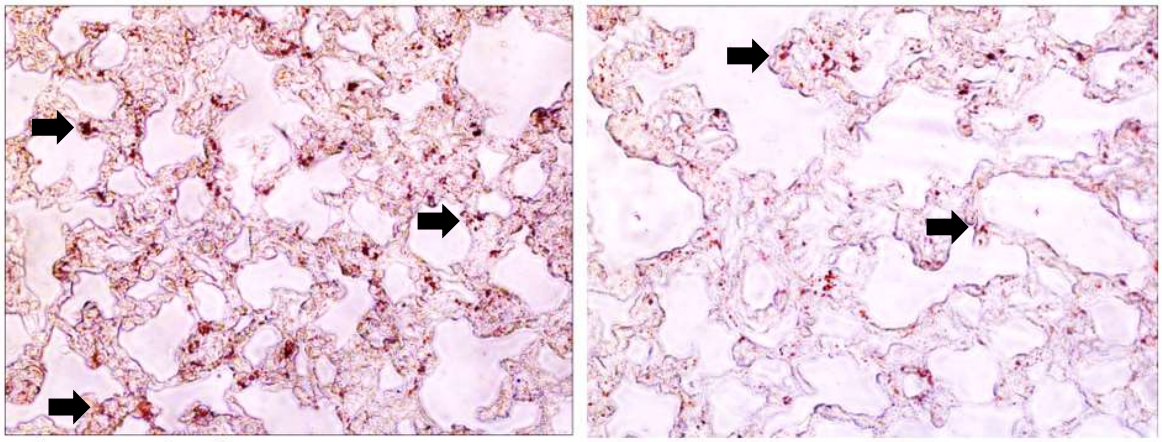
***Figure 7: decreased lipid metabolism gene expression in Tcf21 knockout lungs**. Expression of selected lipid metabolism genes in Tcf21 knockout mice compared to control mice and normalized by GAPDH expression. All mice were induced with tamoxifen at p2 but samples were collected at 3 different time points (p7, p14, and p28). After organ collection qRT-PCR was performed with the isolated RNA of the whole lung. qRT-Pcr was performed in technical triplicates. Standard deviation is shown in error bars. At all time points, a decreased expression of lipid metabolism genes in Tcf21 knockout mice can be observed. Also, the decreased expression of lipofibroblast cell-specific genes indicates a decreased lipofibroblast population in the knockout lungs. Especially, p2p14 time point seems more affected by the loss of Tcf21 at p2 than the earlier or later sample point.*

As seen in Figure 2, the deletion of Tcf21 caused a decrease in the expression of several lipid metabolism genes (Fabp1, Fabp4, Fabp5, Cebp alpha and Cebp beta). This decreased expression level was observed in samples collected at all time points, however, a more severe decrease of the expression levels can be observed with p2p14 time point. Also, p2p7 samples seemed more affected by the loss of

Tcf21 than samples collected at p28. Additionally, expression levels of cholesterol and lipoprotein metabolism genes (LPL, LDLR, and Lcat) are decreased. Again, samples collected at p14 showed higher affection for loss of Tcf21, followed by p2p7 samples. After loss of Tcf21, Plin 2, used as a lipofibroblast marker, gene expression levels were decreased, whereas Col13a1, another lipofibroblast marker, gene expression levels did not seem de- but increased at p2p7 and p2p28 time point. At p2p14 both lipofibroblast genes were expressed less when comparing Tcf21 knockout mice to control mice.

3.4 Amount of neutral lipids is non significantly reduced in Tcf21 knockout lungs

To determine the amount of neutral lipids in 28-day old mutant and control lungs, the whole organ was fixed with 1%PFA and slides with 10µm thick slices were prepared. The tissue then was stained with Oil Red O solution and the excess dye was washed away so only neutral lipid droplets appear red after the staining procedure. While taking pictures at 40x magnification proximal and distal regions were analyzed equally. Figure 3 shows the differences of neutral lipid contents according to area between Tcf21 knockout (mutant) and control lungs.



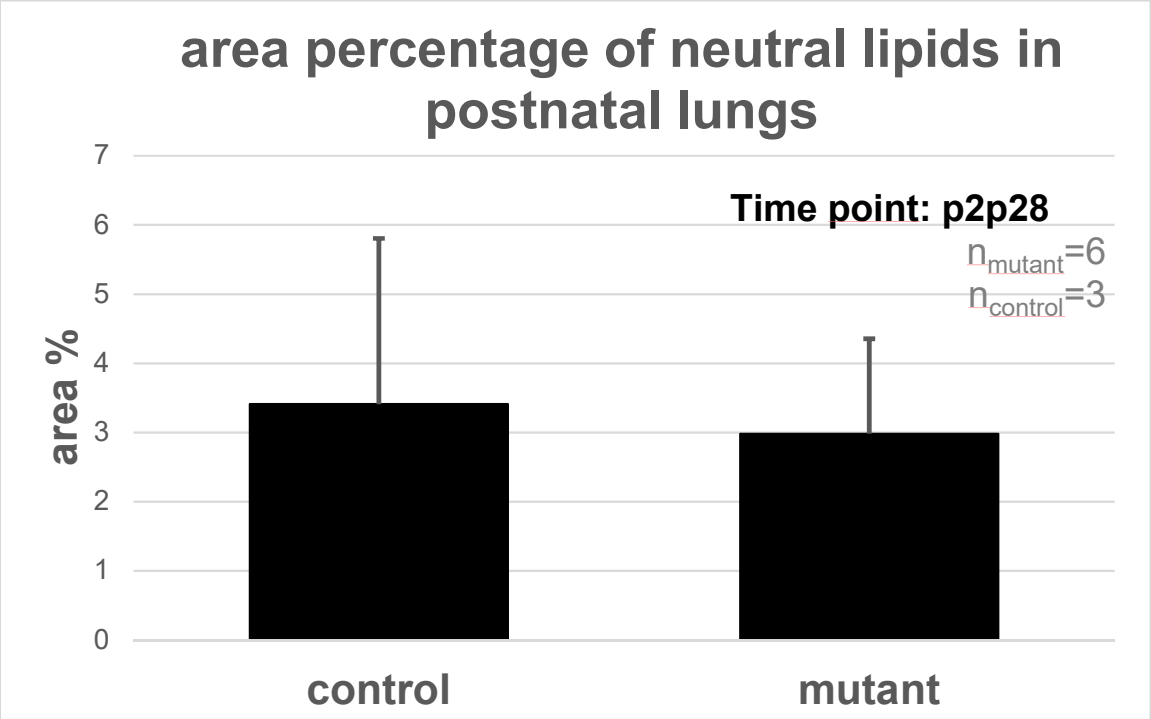
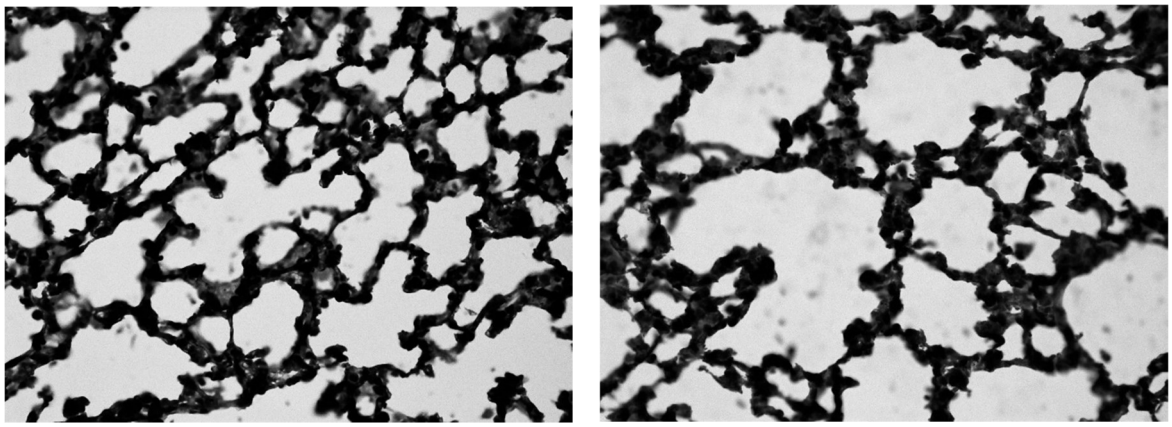


Figure 8: Amount of neutral lipids is slightly decreased in Tcf21 knockout lungs. *Representative picture A and B: stained tissue. Lipid droplets appear red (examples are indicated with arrows). Graph beneath pictures: quantification of all samples. Control and mutant mice were induced with tamoxifen at p2. Lungs were collected at p28, fixed with 1%PFA and embedded. 10µm thick slices were stained with Oil Red O Solution to visualize neutral lipid droplets. Pictures were taken at 40x magnification and the area of red-stained neutral lipid droplets in the lungs was analyzed and plotted. Standard deviation is shown in error bars. In 28-day old Tcf21 knockout mice the area of neutral lipid droplets is slightly but not significantly decreased in comparison to 28-day old control mice.*

Compared to control mice, Tcf21 knockout mice showed a slightly decreased neutral lipid droplet area (Figure 3). However, differences at this time point are not significant.

3.5 Deletion of Tcf21 induces structural changes in postnatal lungs

A haematoxylin and eosin staining was performed to visualize potential structural changes in 4-week old mice after deletion of Tcf21 at postnatal day 2 (Figure 4).



A: control; p2p28; n=3 B: mutant; p2p28; n=6

Figure 9: Tcf21 knockout alveoli show structural differences*. P2p28 control and mutant lungs were collected, fixed and 10µm thick slices were stained with H&E solution to visualize structural changes. Pictures were taken at 40x magnification and analyzed. While control lungs(A) showed fully developed alveoli, lungs missing Tcf21(B) showed impaired alveolarization. Pictures A and B were chosen representatively.*

Tcf21 lacking mice showed less efficient secondary septum formation leading to dilated alveolar spaces. Furthermore, thinning of alveolar walls was observed. No differences regarding the alveolarization intensity between distal and proximal parts of the lung were observable at this time point.

3.6 Surfactant Protein expression and number of alveolar epithelial cells and is reduced in Tcf21 knockout lungs

To determine the effect of postnatal Tcf21 loss on alveolar epithelial cell populations and on the transcription of surfactant proteins, mRNA levels of respective genes in Tcf21 lacking lungs were compared with gene expression in control lungs. Therefore, qRT-PCR was performed, and gene expression levels were normalized by GAPDH expression and the fold change from mutant to control lungs was calculated and plotted (Figure 5).

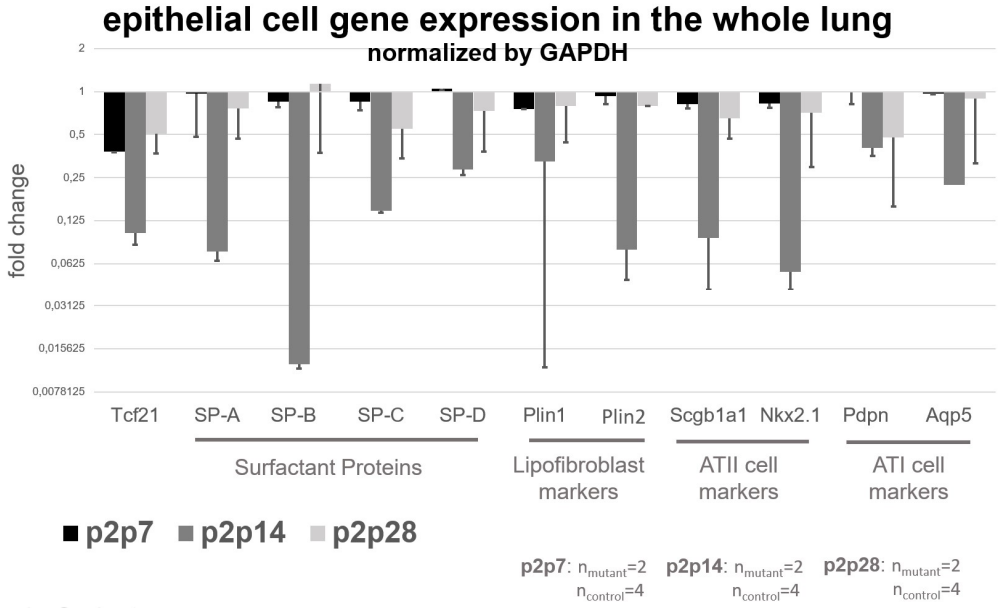


Figure 10: decreased surfactant expression and alveolar epithelial cell population in Tcf21 knockout lungs*. Expression of surfactant protein and epithelial cell-specific genes in Tcf21 knockout mice compared to control mice and normalized by GAPDH expression. All mice were induced with tamoxifen at p2 but samples were collected at 3 different time points (p7, p14, and p28). After organ collection qRT-PCR was performed with the isolated RNA of the whole lung. qRT-Pcr was performed in technical triplicates. Standard deviation is shown in error bars. At all time points, a decreased expression of surfactant protein genes in Tcf21 knockout mice can be observed. Also, the decreased expression of alveolar epithelial and lipofibroblast cell-specific genes indicates a decreased alveolar epithelial cell and lipofibroblast population in the knockout lungs.*

As seen in Figure 5, the deletion of Tcf21 caused a decrease in the expression of surfactant protein genes (SP-A, SP-B, SP-C and SP-D). This decreased expression level was observed in samples collected at all time points, however, a more severe

decrease of the expression levels can be observed with p2p14 time point. Also, p2p28 samples seemed more affected by the loss of Tcf21 than samples collected at p7 regarding the expression levels of surfactant proteins. Additionally, expression levels of pulmonary lipofibroblast specific genes (Plin1 and Plin2) are decreased. Again, samples collected at p14 showed higher affection for loss of Tcf21. After loss of Tcf21, genes that are associated with alveolar epithelial cell population are less expressed. The most severe effect is observed at p2p14 followed by p2p28. Overall, Alveolar Type 2 cell-specific genes (Scgb1a1, Nkx2.1) show a stronger decreased expression level than Alveolar Type 1 cell-specific genes (Pdpn, Aqp5), suggesting that the loss of Tcf21 has a higher impact on the alveolar type 2 cell population than on other alveolar epithelial cells.

4 Discussion

4.1 Deletion efficacy

As seen in figure 1, the deletion of Tcf21 using the Cre-loxP system was effective for all time points. However, activating this system with intragastric tamoxifen induction at postnatal day 2, the generation of conditional somatic mouse mutants is not guaranteed. The efficacy of this activation method relies strongly on the person proceeding the injection. Coming in contact with intragastric injections the first time, I hardly never achieved to peruse a deletion effect of 100%.

Furthermore, Tcf21 expression may no be to Tcf21mCrem recombination, even with perfectly administrated tamoxifen induction. Potentially, cells have to show a certain level of Tcf21 promoter activity to express enough mCrem so Cre recombination is possible upon the injection of tamoxifen. Thus, we possibly were not able to detect cells which expressed Tcf21 in lower levels only.

4.2 Problems with primer annealing

When checking the melting curves after running qRT-PCR we discovered that primer annealing for Tcf21 did not work sufficiently in p2p14 samples. Hence, we changed the primer dimers when working with the other time points (p2p7 and p2p28). However, due to time limitations, it was impossible to repeat the setup with one primer pairs for p2p14 samples. Thus, qRT-PCR data obtained at the p2p14 time point are not totally reliable, nevertheless, their tendencies can be taken into considerations.

4.3 Decreased lipid metabolism after deletion of Tcf21

Preliminary data showed that the Tcf21 lineage constitutes to the lung lipofibroblast population and the accumulation of lipid droplets in lipofibroblasts is regulated by Tcf21 expression. Hence, a subset of several genes associated with lipid metabo-

lism and lipoproteins was chosen to be analyzed after the loss of Tcf21. As expected, the expression level of the whole subset was decreased in Tcf21 lacking lungs. For example, the expression of transcription factors CCAAT/enhancer-binding protein (Cebp) alpha, and Cebp beta, which play an important role during adipogenesis and are required for the maintenance of lipid storages and energy homeostasis postnatally, was decreased in Tcf21 lacking lungs.[25-27] But not only glucose and lipid metabolism genes were less expressed after deletion of Tcf21, but also the expression of cholesterol metabolism genes like LDLR, and LPL was decreased. The low-density lipoprotein (LDL) receptor (LDLR) mediates the uptake of LDL for cholesterol synthesis whereas the enzyme lipoprotein lipase (LPL) has its crucial role in lipoprotein metabolism since it hydrolyzes triglycerides in lipoproteins and is also known to be a ligand-bridging factor for receptor-mediated uptake of lipoproteins. [28, 29]

Expression levels of lipid metabolism genes seem more affected by the loss of Tcf21 if samples are collected after one week (p2p7) compared to samples collected after 4 weeks (p2p28). This indicates an increased lipid metabolism in postnatal murine lungs during the early stage of alveolarization, peaking in the second postnatal week. [1, 30, 31]

using qRT-PCR as analyzing method, only the expression on mRNA level was analyzed, however, this does not for sure imply that also the mice's phenotype is affected by fold-changes in mRNA expression. It cannot be guaranteed that the decreased expression is significant for every gene. Some genes are expressed in high ratios under normal conditions. For those genes, a small reduction of mRNA expression will not cause severe differences in the protein expression level. On the other hand, genes that are expressed in low ratios only may be more affected even by small changes. Furthermore, protein expression and activity are not only regulated by mRNA expression but also by posttranslational modifications like esterification or phosphorylation.

4.4 Decreased lipofibroblast population in Tcf21 knockout lungs

As shown in figure 2 and 5, a decrease in the expression of genes that are used as lipofibroblast cell markers was noticeable. Reduced expression levels of plin 1 and plin 2 indicate a decrease in the pulmonary lipofibroblast population upon deletion of Tcf21. This salience is in compliance with findings from former obtained data.[1] Plin 2, which is also known as adipose differentiation-related protein (ADFP) is used to differentiate between lipofibroblasts and other types of fibroblasts. Plin2 plays an important role during neutral lipid droplet formation and is believed to be mainly responsible for the storage of neutral lipids in pulmonary lipofibroblasts and the subsequent transfer of neutral lipids to AT2 cells for surfactant biosynthesis.[32, 33]

Since the expression levels of Col13a, which is used as a fibroblast marker, were not reduced, one can assume that the population of pulmonary fibroblasts is not affected by the loss of Tcf21. [34]

4.5 Neutral lipid content may be affected by loss of Tcf21 at earlier alveolarization stage

During this project, the differences in neutral lipid accumulation in late-stage alveolarization murine lungs after postnatal loss of Tcf21 was observed. It can be stated that the loss of Tcf21 at postnatal day 2 does not has significant effects on the neutral lipid contents until the age of 4 weeks.

Since it is known, that triglyceride (neutral lipid) content in the lung peaks during early alveolarization stage, to be more precise during the second postnatal week in mice, it may be possible that at the age of 4 weeks, the neutral lipid content in the lungs is reduced anyways and therefore, loss of Tcf21 is not severe at this time point.[1, 30, 31] Additionally, other cell types could compensate for the loss of pulmonary lipofibroblasts by partially overtaking their functions. Comparing the amounts of neutral lipids in postnatal lungs at earlier time points is still in the focus of our lab group.

4.6 Decreased surface area in Tcf21 knockout alveoli

Figure 4 shows that the postnatal loss of Tcf21 induces structural changes in the lung. Impaired alveolarization is defined by thinning of alveolar walls as well as impaired secondary septum formation leading to dilated alveolar spaces. Hence, Tcf21 knockout lungs show decreased alveolar surface due to hypoplastic structures. One can assume that these impairments affect the gas exchange and metabolism in Tcf21 lacking animals. Currently, further investigations regarding this hypothesis are conducted.

4.7 Reduced alveolar epithelial cell populations

Our data show that the loss of Tcf21 leads to a decrease in alveolar epithelial cell population. However, it is remarkable that AT2 cells seem to be more affected by the loss of Tcf21 than AT1 cells.

Whereas the flat and thin AT1 cells build the blood-air barrier, thus facilitate gas exchange in the lung, cubical AT2 cells are mainly responsible for surfactant production. In humans, AT2 cells are first detected around week 24 of gestation. By week 34 of gestation, most AT2 cells have differentiated and surfactant production is sufficient, enabling the new born's alveoli to expand and fill with air during the very first breath and preserving the infant from developing respiratory distress syndrome.[6] TGF-β signaling plays an important role during the differentiation process of AT2 cells. But TGF-β signaling is also important for alveolarization, where dysregulation of this pathway, either up- or down-regulation, means hindrance of successful alveologenesis.[6]

Being able to differentiate into AT1 cells if needed, AT2 cells play a crucial role in tissue repair. But also during cellular homeostasis since the trans-differentiation from AT2 to AT1 cells occurs all naturally during lung development.[2, 10] Just as differentiation of AT2 cells is regulated by TGF-β, their trans-differentiation into AT1 cells is linked to TGF-β/Smad2,3,4 signaling.[6] Furthermore, it is presumed that the late stage microRNA (miR)375, which is expressed highest shortly before and after birth, inhibits β-catenin signaling without which AT2 cells remain as such, implying

that loss of miR375 would lead to increased trans-differentiation. This hypothesis was established on data generated by Wang et al in 2013, who observed that a decreased miR375 level induces increased β-catenin signaling which then promotes the trans-differentiation from AT2 to AT1 cells in vitro.[2, 10] Hence, it could be that both types of alveolar epithelial cells are affected by loss of Tcf21 equally at first, but since the lung is missing AT1 cells, remaining AT2 cells transdifferentiate into AT1 cells, making up for the primary loss of AT1 cells but reducing the population of AT2 cells even more.

4.8 Reduced expression of surfactant proteins

Since surfactant is mainly produced by AT2 cells and as stated above, the AT2 cell population is decreased in Tcf21 knockout lungs, it is obvious that also the expression of surfactant proteins is reduced in these lungs. Expression levels differ more severe at later time points (p2p28) since surfactant production peaks postnatally, therefore, also control mice need some weeks to establish a surfactant pool which allows showing differences between control and mutant mice.

Especially surfactant protein C (SP-C) seems to be more affected than the other surfactant proteins which is most probably due to the fact that SP-C is only translated in AT2 cells whereas other surfactant proteins are also synthesized by other cell types such as clara cells.

4.9 Outlook

This thesis helped to provide further insights into the role of Tcf21 during postnatal lung development, especially on its importance in lipid metabolism and surfactant synthesis. Nevertheless, some open questions remain which our lab will focus on. Further investigations are needed to determine the effect Tcf21 has on alveolar epithelial cells and to confirm or disprove the transdifferentiation of AT2 cells to AT1 cells upon Tcf21 loss. Other aspects of future research deal with the efficiency of lipid transport between Lipofibroblasts and AT2 cells and if more efficient lipid transport could restore normal surfactant production.

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