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The Ferocious Role of Iron in Alveolar Type II Epithelial Cell Function

Thesis submitted for the degree of Doctor of Philosophy

Trinity College Dublin

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university, has been completed by me, Sarah Kenny, under the guidance of Professor Suzanne Cloonan. This thesis is entirely my own work, with the exception of the following figures;

Chapter 3:

Figure 3.9B - This work was carried out by Li-Chao Fan^{1,2} (University of Cornell)

Figure 3.7A-D, Figure 3.10, Figure 3.11 and Figure 3.12 - These samples were harvested by Li-Chao Fan^{1,2} (University of Cornell). For the lipidomics datasets presented in Figures 3.7, 3.10, and 3.11, I performed the data mining, all downstream analyses, and figure preparation. For Figure 3.12, I processed the frozen lung samples, optimized the Pro-SFTPC antibody staining protocol, and conducted the subsequent analyses.

Chapter 4:

Figure 4.11 D - This work was carried out by Ziling Huang^{1,2} (University of Cornell)

Chapter 5:

Figure 5.1 and 5.4 - This work was carried out by Ziling Huang^{1,2} (University of Cornell)

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Her love and sweet face,
Seven years, her warm embrace,
In my heart, her place.

I dedicate this thesis to you in your memory.

Summary

Alveolar type 2 (AT2) cells are specialized epithelial cells in the lung with critical roles in maintaining alveolar function and integrity. These metabolically active cells are primarily responsible for synthesizing, secreting, and recycling pulmonary surfactant; a complex mixture of lipids and proteins that reduces surface tension at the air-liquid interface. Beyond surfactant production, AT2 cells are pivotal in lung repair, possessing the ability to self-renew or transdifferentiate into alveolar type 1 (AT1) cells to restore epithelial integrity following injury. AT2 cells are central to alveolar health, with loss or dysfunction of AT2 cells contributing to several chronic lung diseases.

The functional identity of AT2 cells is intimately linked to mitochondrial homeostasis, reflected by their reliance on mitochondrial activity to sustain the metabolic demands of their specialized roles. Mitochondria require a variety of nutrients for optimal function, with iron being one of the most essential. Mitochondrial energy production and iron metabolism are interdependent processes, each crucial to the other's function. Together, they lie at the centre of cellular and tissue iron homeostasis and both have been implicated in the pathogenesis of several chronic lung diseases. Yet mitochondrial iron (MITOIRON) regulation is an underappreciated and understudied function in AT2 cells of the lung. We **hypothesized** that iron metabolism, specifically MITOIRON metabolism influences AT2 cell-specific functions and contributes to their dysfunction in disease.

In this thesis this hypothesis was tested using both *in vitro* and *in vivo* models, whereby iron and MITOIRON levels were manipulated in AT2 cells and their effects on surfactant homeostasis and alveolar repair examined. Collectively, these approaches revealed that both excessive and insufficient iron levels negatively impact AT2 cell function, with modulation of MITOIRON particularly detrimental. Specifically, disruptions in MITOIRON metabolism in AT2 cells profoundly impaired all aspects of surfactant production and AT2 cell mediated repair processes both *in vitro* and *in vivo*. CRISPR/Cas9-mediated knockdown of the mitochondrial iron transporters Mitoferrin 1 (*Mfrn1*) and Mitoferrin 2 (*Mfrn2*), significantly decreased intracellular iron, heme and MITOIRON levels *in vitro*. Reduced MITOIRON levels stunted wound healing and impaired surfactant synthesis, exocytosis and uptake *in vitro*. Conditional loss of *Mfrn1/2* in murine AT2 cells *in vivo* resulted in a loss of AT2 cell markers and disruptions in surface tension. Additionally, these mice presented with severe alveolar neutrophilia, inflammation and failed to activate the integrated stress response, leading to lung-iron dyshomeostasis, acute lung injury, a loss of lung integrity and ultimately mortality. In the above models, such AT2 cell iron deficiency unexpectedly correlated with the presence of a senescent-like phenotype, a recognized characteristic of AT2 cells in lung disease but commonly linked to excessive iron accumulation. The development of an *in vitro* model of the

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injured lung epithelium using bleomycin (BLM) or cigarette smoke (CS) extract revealed that AT2 cell iron-deficiency is coupled with pre-senescent characteristics. This may act as a trigger for AT2 cells to acquire more iron, leading to AT2 cell iron accumulation and sustained cellular senescence. These findings were recapitulated in a murine BLM-induced fibrosis model whereby AT2 cells iron deficiency precedes elevated senescence markers and iron accumulation.

This thesis provides the most in-depth analysis of how iron and MITOIRON metabolism contributes to the specialized functions of AT2 cells, including the first-ever targeted genetic manipulation of MITOIRON specifically in AT2 cells. Together, the findings uncover a previously unrecognized role for iron in sustaining AT2 cell function, implicating MITOIRON metabolism as a potential contributor to cellular dysfunction in lung disease. By illuminating the link between mitochondrial iron and AT2 cell integrity, this work opens new avenues for therapeutic intervention in disorders marked by AT2 cell dysfunction and senescence.

Lay Summary

Our lungs contain tiny air sacs called alveoli, essential for breathing. Within these air sacs are specialized cells called alveolar type 2 (AT2) cells. These cells play important roles in keeping the lungs healthy. They produce a special substance called surfactant, which helps keep air sacs open, making breathing easier. Additionally, AT2 cells can repair the lungs by either replacing themselves or turning into another cell type (AT1 cells) to rebuild damaged areas. Because of their critical roles, problems with AT2 cells can lead to serious lung diseases.

Inside each AT2 cell are mitochondria, the “powerhouses”, that supply energy needed for surfactant production and cell repair. The way mitochondria handle energy production is closely linked to iron metabolism; with each process supporting the other. Iron is an essential mineral in our bodies, and proper iron balance is necessary for good health. However, when iron levels become unbalanced in the lung, this can potentially contribute to lung diseases. Despite this connection, it is unclear exactly how iron and specifically mitochondrial iron (MITOIRON) affects AT2 cell function.

In this study, we investigated how iron imbalance, particularly MITOIRON, affects AT2 cells using cell experiments in a laboratory (*in vitro*) and studies in animals (*in vivo*). We found that both too much and too little iron negatively impact AT2 cells. Excess iron stopped cells from effectively taking up surfactant materials, while insufficient iron disrupted their ability to release surfactant. Both high and low iron levels also impaired AT2 cells' ability to heal injuries.

Notably, when we experimentally blocked the transport of iron into mitochondria (by reducing two important iron transport proteins, Mitoferrin 1 and Mitoferrin 2, AT2 cells were severely affected and were unable to activate the proper stress responses. These cells struggled to produce and handle surfactant, and their ability to repair injuries dramatically declined. In animal experiments, mice with reduced mitochondrial iron transport specifically in AT2 cells, developed lung inflammation, damage, and respiratory failure, underscoring how critical MITOIRON is for AT2 cells and healthy lungs.

Surprisingly, we observed that a lack of iron was linked to AT2 cells becoming senescent, a state that reduces their ability to function normally which is also associated with lung disease. We created models simulating lung injury by treating cells with damaging substances like cigarette smoke or chemicals. Initially, after this damage, AT2 cells lost iron and showed early signs of senescence. These results were mirrored in mouse models of injury and fibrosis, suggesting that iron loss could be an early factor driving AT2 cell dysfunction in lung injury and disease.

Overall, this research uncovers a new and critical role for iron, especially MITOIRON, in maintaining AT2 cell function. It provides important insights into how iron dysregulation can

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contribute to lung diseases. These findings highlight mitochondrial iron as a promising target for future treatments, potentially restoring AT2 cell function and slowing lung disease progression.

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Abbreviations

ACO1	Aconitase 1
ACO2	Aconitase 2
ADGRF5	Adhesion G-protein coupled receptor F5
ALA	5-Aminolevulinic acid
ALI	Acute lung injury
AM	Alveolar Macrophages
APS	Ammonium persulphate
AT1	Alveolar type I epithelial cell
AT2	Alveolar type II epithelial cell
ATP	Adenosine triphosphate
ATP-citrate	Acyl
BALF	Bronchoalveolar lavage fluid
BLM	Bleomycin
BMP	Bone morphogenetic protein
CCL2	C-C Motif Chemokine Ligand 2
CCL5	C-C Motif Chemokine Ligand 5
cDNA	Complementary DNA
CDP-choline	Cytidine diphosphocholine
CER	Ceramide
CFH	Cell-free hemoglobin
CIA	Cytosolic Iron-Sulfur Assembly Component
COPD	Chronic Obstructive Pulmonary Disease
CS	Cigarette smoke
CSE	Cigarette smoke extract
CXCL2	C-X-C Motif Chemokine Ligand 2
CXCL5	C-X-C Motif Chemokine Ligand 5
CXCL7	C-X-C Motif Chemokine Ligand 7
CXCL8	C-X-C Motif Chemokine Ligand 8 (IL-8)
DAG	Diacylglycerol
DCYTB	Duodenal Cytochrome B
DFP	Deferiprone
DFX	Deferoxamine
DHAP	Glucose-derived dihydroxyacetone phosphate
DMT1	Divalent metal transport
DPPC	Dipalmitoylphosphatidylcholine

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ECM	Extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ETFDH	Electron transfer flavoprotein dehydrogenase
FA	Fatty Acids
FAC	Ferric ammonium citrate
FAO	Fatty acid oxidation
FASN	fatty-acid synthase
FBS	Fetal bovine serum
FBXL5	F-Box and Leucine-Rich Repeat Protein 5
Fe ²⁺	Ferrous
Fe ³⁺	Ferric
FeCH	Ferrochelataase
Fe-S	Fe-sulfur
FGF10	Fibroblast growth factor 710
FGF7	Fibroblast growth factor 7
FGFR2	Fibroblast growth factor receptor 2
FLVCR	Feline Leukemia Virus Subgroup C Receptor
FPN	Ferroportin
FTH	Ferritin Heavy Chain
FTL	Ferritin Light Chain
GFAAS	Graphite furnace atomic absorption spectrometry
GFP	Green fluorescent protein
HAMP	Hepcidin
HBP	Heme binding proteins
HCP1	Heme carrier protein 1
HFE	Homeostatic Iron Regulator
HIF	Hypoxia inducible factors
HMB	Hydroxymethylbilane
HO	Heme oxygenase
HRG1	Heme Responsive Gene 1
HRI/ EIF2AK1	Heme regulated inhibitor
IL1 α	Interleukin 1 Alpha
IL1 β	Interleukin 1 Beta
IL6	Interleukin 6

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ILD	Interstitial lung disease
IMM	Inner mitochondrial membrane
IPF	Idiopathic Pulmonary Fibrosis
iPSCs	Induced pluripotent stem cells
IRE	Iron regulatory element
IRP1	Iron regulatory protein 1
IRP2	Iron regulatory protein 2
ISC	Iron-Sulfur Cluster Assembly
ISR	Integrated stress response
LBP	lipopolysaccharide binding protein
LCN	Lipocalin
LIP	Labile iron pool
LPS	Lipopolysaccharide
MCU	Mitochondrial calcium uniporter
MFRN1	Mitoferrin 1
MFRN2	Mitoferrin 2
MHCII	Major histocompatibility complex class II
MITOIRON	Mitochondrial iron
MMPs	Matrix metalloproteinases
MRP-5	Multidrug resistance-associated protein 5.
mtDNA	Mitochondrial DNA
MTFT	Mitochondrial ferritin
mtROS	Mitochondrial reactive oxygen species
MVBs	Multivesicular bodies
NADH	Nicotinamide Adenine Dinucleotide (Hydride)
NCOA4	Nuclear receptor coactivator 4
NTBI	Non transferrin bound iron
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PA	Phosphatidic acid
PBG	Porphobilinogen
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PGRMC2	Progesterone receptor membrane component 2
PHD	Prolyl hydroxylase domain

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PI	Phosphatidylinositol
PKC	Protein kinase C
PL	Phospholipid
PPIX	Protophyrin IX
PS	Pulmonary surfactant
RBC	Red blood cells
RIN	RNA integrity number
ROS	Reactive oxygen species
RT-QPCR	Real time quantitative polymerase chain reaction
SASP	Senescence-associated secretory phenotype
scRNA-seq	Singl-cell RNA sequencing
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFTP-A	Surfactant Protein A
SFTP-B	Surfactant Protein B
SFTP-C	Surfactant Protein C
mSFTPC	Mature Surfactant Protein C
SFTP-D	Surfactant Protein D
SLC02B1	Solute Carrier Family 2 Member B1 (Glucose transporter)
STEAP	Six transmembrane epithelial antigen of prostate
TBI	Transferrin bound iron
TCA	Tricarboxylic acid cycle
TEMED	N,N,N',N' Tetramethylethylenediamine
TF	Transferrin
TFR/CD71	Transferrin receptor
TGFβ	Transforming growth factor β
TLR	Toll-like receptors
TNF	Tumor Necrosis Factor
UROD	Uroporphyrinogen decarboxylase
WNT	Wingless-related integration singalling
ZIP14	Zinc Transporter ZIP14
ZIP8	Zinc Transporter ZIP8

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1. Introduction

In lung biology AT2 cells emerge as the protagonists of alveolar maintenance and homeostasis. These metabolically active cells are primarily responsible for synthesizing, secreting, and recycling pulmonary surfactant; a complex mixture of lipids and proteins that reduces surface tension at the air-liquid interface, facilitating efficacious gas exchange (3, 4). Beyond surfactant production, AT2 cells are pivotal in lung repair. They possess the ability to self-renew and/or transdifferentiate into AT1 cells, thereby restoring epithelial integrity following injury (5, 6). Moreover, AT2 cells act as immune sentinels, secreting antimicrobial factors that directly neutralize pathogens and activate nearby immune cells (4, 7). The critical role of AT2 cells is further evident in lung disease, where their dysfunction or loss contributes to the development and progression of various acute and chronic lung conditions (8). These roles highlight the need to further study and unravel the molecular cues regulating their function in both health and disease.

1.1 AT2 cells in biology

Alveoli, residing at the terminal of airways, are the main units for gaseous exchange. AT1 and AT2 cells constitute the interior structure of the alveolus (**Figure 1.1**) (4).

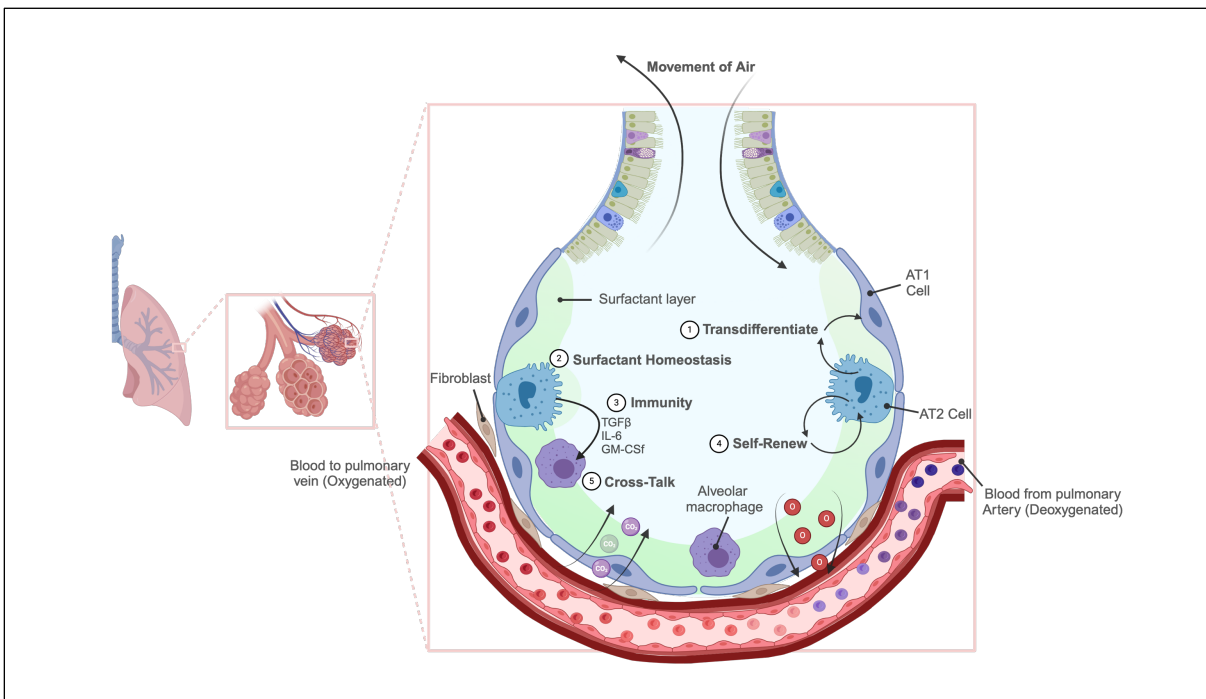


Figure 1.1: Alveoli structure, with emphasis on AT2 cells and their key functions in the alveoli

(1) Trans-differentiation, the process of AT2 cells turning into AT1 cells. (2) Surfactant homeostasis, including proper secretion, storage, synthesis and turnover of surfactant in the alveolar space. (3) Immunity, secreted immunological factors that stimulate nearby immune cells. (4) Self-Renew, the process of AT2 replacement by AT2 cells. (5) Cross-talk, AT2 cells are central to alveolar homeostasis and communicate with resident cells through their secreted factors.

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The exterior of the alveolus wall mainly consists of capillary endothelial cells and interstitial cells, including fibroblasts. AT1 cells, covering approximately 95% of the alveolar surface, are thin in shape and facilitate gaseous exchange across the alveolar membrane (4). AT2 cells, constituting the remaining 5% of the surface, are cuboidal in shape and serve as critical regulators of alveolar homeostasis. Their key functions in (a) surfactant homeostasis, (b) immunity and (c) alveolar repair and maintenance positions them at the centre of alveolar and lung health.

1.1.1 Surfactant Homeostasis

AT2 cells are the primary producers, secretors and recyclers of pulmonary surfactant (PS); a crucial component in maintaining lung homeostasis by regulating alveolar surface tension and enabling efficient inflation and deflation (3). PS is a highly specialized mixture of lipids and surfactant-associated proteins, optimized for its biophysical and immunological functions.

(a) Composition

Lipids comprise approximately 90% of PS, with phospholipids (PLs) accounting for 80% of the total lipid fraction. Of these, phosphatidylcholine (PC) species dominate (~85%), primarily in their saturated form as dipalmitoylphosphatidylcholine (DPPC, 16:0/16:0 PC) which is the primary lipid responsible for surfactant surface activity (9, 10). While DPPC is essential for reducing alveolar surface tension, a balance with other saturated and unsaturated PC species is necessary to optimize surfactant absorption and film formation at the air-liquid interface (9). Acidic phospholipids, particularly phosphatidylglycerol (PG), constitute ~15% of the pulmonary surfactant phospholipid pool. While their precise biophysical role remains unclear, they have previously been shown to play a role in immunity (11). Other lipid components, including cholesterol, glycolipids and free fatty acids, further contribute to surfactant composition (3).

The remaining 10% of PS consists of four surfactant-associated proteins (SFTP-A, SFTP-B, SFTP-C, and SFTP-D), which interact with the lipid fraction to regulate surfactant. SFTP-B and SFTP-C are highly hydrophobic, playing a critical role in surfactant biophysical properties by facilitating PS spreading and stability (3, 9). In contrast, SFTP-A and SFTP-D are hydrophilic members of the collectin family, primarily involved in innate immunity and inflammation. This highlights the dual role of PS in both biomechanical lung function and host defence (3, 12).

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(b) Synthesis

In AT2 cells, lipid synthesis for pulmonary surfactant involves a complex interplay of biochemical pathways primarily occurring in the endoplasmic reticulum (ER), mitochondria and peroxisomes (13). To acquire the lipids needed for PS AT2 cells can either synthesize them or uptake them from the microenvironment. The synthesis of PS-lipids is highly dependent on the availability of fatty acids (FA), enzymes including fatty-acid synthase (FASN), ATP-citrate lysase (ACYL) and acetyl CoA carboxylase catalyse the biosynthesis of surfactant lipids in the lung (14). Additionally, hormonal, cytokine and growth factor regulation can enhance surfactant synthesis through these enzymatic pathways (15).

Key intermediates in this lipid synthesis include phosphatidic acid (PA) and diacylglycerol (DAG), which are generated via *de novo* pathways from glucose-derived dihydroxyacetone phosphate (DHAP). PA serves as a vital precursor by transforming into either phosphatidylglycerol (PG) and phosphatidylinositol (PI), or further into DAG, which subsequently contributes to the synthesis of phosphatidylcholine (PC) (16). PC biosynthesis predominantly follows the cytidine di-phosphocholine (CDP-choline) or Kennedy pathway, initiated by the dietary uptake of choline. Choline cannot be synthesized *de novo*, making the regulation of PC biosynthesis directly dependent on dietary choline availability (17). Choline is then phosphorylated and converted into CDP-choline, which combines with DAG to form PC.

As previously mentioned the majority of PC species are found as DPPC. Interestingly studies suggest approximately 45% of DPPC in AT2 cells is formed via *de novo* synthesis. The remainder ~60% is generated by the deacylation and reacylation of available phospholipids (18), highlighting the unique adaptability of AT2 cells. Other lipid components in pulmonary surfactant exhibit similar biosynthetic patterns (13). Under substrate limiting conditions, AT2 cells can adapt accordingly to produce surfactant phospholipids (PL), though the relative proportions of these lipids may vary (9, 14, 17, 19). Such imbalances can lead to altered surfactant function, underscoring the critical importance of maintaining regulated and balanced lipid synthesis to prevent pathological disruptions in lung physiology.

Finally, surfactant proteins are all synthesized in AT2 cells but not exclusively with club cells and alveolar macrophages (AM) showing affinity to surfactant proteins A, B and D (20, 21).

(c) Secretion

Surfactant secretion is a complex dynamic process that relies heavily on environmental and mechanical cues. Secretion of PS is largely regulated by the exocytosis of lamellar bodies. Lamellar bodies serve as the storage hub for PS, which, upon stimulation, fuse with the plasma membrane of AT2 cells to release PS into the alveolar space (3). To date the majority of surfactant secretion research has utilized radiolabelled surfactant lipids. *In vitro* studies have shown that triggers such as beta-adrenergic agonists, adenosine triphosphate (ATP) and ionophores activate cAMP and protein kinase C (PKC), coupled with calcium influx, to directly or indirectly facilitate PS exocytosis (22-24). *In vivo*, surfactant secretion is also regulated by biophysical processes which increase secretion through mechanical stretching and hormonal signalling, with beta-adrenergic signalling thought to mediate these effects (25). Similarly, mechanical stretch in cultured primary AT2 cells stimulates Ca^{2+} influx (26) and upregulates surfactant genes (27), followed by the exocytosis of radio-labelled PC (26), which suggests surfactant secretion is being facilitated.

Surfactant protein secretion depends on the specific protein being secreted. SP-A and SP-D are secreted continuously by AT2 cells, as they are minimally present in lamellar bodies and SFTP-A levels do not increase when AT2 cells are stimulated with secretagogues (28, 29). In contrast, SFTP-B and SFTP-C constitute a large proportion of proteins in lamellar bodies, suggesting they are secreted as part of PS exocytosis (29).

(d) Recycling

Secreted pulmonary surfactant is predominantly cleared by AT2 cells through their unique endocytic capacity (30-32). Additional clearance mechanisms include degradation within the alveolar space, lymphatic drainage and macrophage catabolism (3). Similar to surfactant secretion, research into surfactant clearance relies heavily on radiolabelled surfactant lipids and their precursors. A notable limitation of these studies is that radiolabelled lipids may not fully replicate the behaviour of native pulmonary surfactant lipids. Nevertheless, evidence demonstrates that radiolabelled surfactant lipids instilled intratracheally are incorporated into AT2 cells and subsequently into lamellar bodies, suggesting endocytic uptake followed by turnover for future secretion (33).

AMs also ingest and degrade surfactant phospholipids, releasing degraded surfactant products extracellularly (34). However, due to their uneven distribution and lower abundance relative to AT2 cells, macrophages contribute minimally to overall surfactant

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clearance under normal physiological conditions, estimated at approximately 8–20% (30, 32).

SFTP-A plays a critical role in lipid clearance from the alveolar space, primarily through receptor-mediated endocytosis of SFTP-A:DPPC complexes by AT2 cells (35). Following internalization, SFTP-A:DPPC traffics through the cell, undergoing partial recycling and intracellular storage, with lipids localizing to multivesicular bodies; although precise intracellular pathways remain partially unresolved (36). Additionally, AMs also contribute to SFTP-A clearance, albeit at slower rates compared to DPPC (36). *In vitro* studies suggest AT2 cells and AMs internalize SFTP-D, but their precise role and mechanism in physiological conditions remain undefined and require further investigation (37, 38).

Intratracheal instillation experiments with radiolabelled SFTP-C or SFTP-B demonstrate efficient internalisation of both proteins into lamellar bodies. Interestingly, SFTP-C internalization occurred more rapidly than labelled DPPC, whereas the opposite was true for SFTP-B, which accumulated more in AMs (33, 39). Together, these findings indicate a high turnover rate for SFTP-C by AT2 cells, while SFTP-B clearance shows a higher affinity for AMs.

1.1.2 Immunity

The lung is continuously exposed to exogenous irritants such as dust, micro-organisms, vapours, fumes and metal particulates. Despite this exposure, chronic inflammation is uncommon due to the lung's innate immune system. This system includes antimicrobial molecules, structural barriers, mechanical defences and phagocytic cells (4). In the alveoli, the initial defence is provided by the alveolar epithelium, primarily composed of AT1 and AT2 cells. While AT1 cells primarily function as physical barriers, AT2 cells play a pivotal role in orchestrating alveolar immunity (12).

AT2 cells secrete a wide variety of anti-inflammatory and anti-microbial molecules, including antioxidants, collectins, cathelicidin and β -defensins (4, 12). AT2 utilise these molecules to modulate immune cell activity and dampen inflammation under normal conditions (40-42). During infection, AT2 cells can elicit an immune response and secrete cytokines (IL-1 β , IL-1 α , TNF, and IL-6) and chemokines (GRO (CXCL1), ENA-78 (CXCL5), IL-8 (CXCL8), MIP-2 (CXCL2), NAP-2 (CXCL7), RANTES (CCL5), and MCP-1 (CCL2)) (12, 43).

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AT2 cells engage with both arms of the immune system through their secretome, antigen presentation or cell-to-cell communication. *In vitro* studies indicate that AT2 cells mount an immediate pro-inflammatory response upon pathogen exposure (44). Activation of type I interferon signalling pathways in AT2 cells is crucial for the activation of CCR2⁺ monocytes and their subsequent differentiation into interstitial macrophages (IMs) in mice (45). This underscores AT2 cells role as primary rapid responders, eliciting downstream effects in the immune response to invading pathogens.

Secreted collectins, SFTP-A and SFTP-D, enhance the phagocytic capabilities of AMs either directly by stimulating AMs or indirectly by binding pathogens to facilitate phagocytosis (46). Furthermore, surfactant proteins modulate the inflammatory response through suppression of LPS-induced inflammation *in vivo* (47, 48). Mice deficient in SFTP-A present with decreased bacterial clearance coupled with increased inflammation and inflammatory cell recruitment during infection (46). One explanation could be SFTP-A role in mitigating inflammation by suppressing the production of pro-inflammatory factors and blocking IFN- γ receptor interactions in AMs (42). Moreover, bacterial uptake in AMs from mice lacking SFTP-A (SP-A^{-/-}) or SFTP-D (SP-D^{-/-}) is impaired (46). As mentioned both SFTP-A and SFTP-D are also associated with AMs and club cells (20, 21), therefore the specific contributions of AT2-derived surfactant proteins to these processes remain to be elucidated. Nonetheless, these proteins evidently play a crucial role in the lungs immune response to infection.

Additionally, AT2 cells secrete lipopolysaccharide binding protein (LBP), which interacts and binds with lipopolysaccharide (LPS) (49). AT2 cells express toll-like receptors (TLRs) on their surface, which facilitate the interaction of LPS:LBP complexes with specific receptors on leukocytes (4). Other key alveolar proteins secreted by AT2 cells involved in innate immunity include lactoferrin, which sequesters iron from bacteria and defensins, which are antimicrobial peptides (50).

The role for AT2 cells in the adaptive immune response is debated. AT2 cells are characterised as expressing major histocompatibility complex class II (MHCII), though at levels lower than those found in classical antigen presenting cells (51). *In vitro* studies suggest that AT2 cells are less effective at stimulating certain T-cell subsets. However, targeted MHCII deletion in AT2 cells results in moderately exacerbated outcomes in viral disease models in mice, suggesting a likely significant role for AT2 cells in T-cell activation (52).

Recent studies have shown that AT2 cells can leverage their nutritional status to combat invading pathogens through a mechanism known as nutritional immunity. This emerging

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concept describes how immune cells adapt their metabolome to deprive pathogens of essential nutrients. *In vitro* AT2-like cells have been observed to modify their iron metabolism differently based on whether the pathogen is intracellular or extracellular (53).

Together these results showcase AT2 cells' adaptability and pivotal role in both direct pathogen response and coordination of the immune system's overall defence mechanism.

1.1.3 Alveolar Repair and Maintenance

The differentiation and regeneration of alveolar cells is regulated by complex interactions between AT2 cells and their surrounding environment. Following injury, AT2 cells release autocrine and paracrine signals that trigger the production of inflammatory cytokines and growth factors. External factors (inflammatory signals, growth factors, nutrient availability, mechanical tension) and internal factors (metabolism) modulate signalling pathways in AT2 cells (54-61), which collectively guide AT2 cells' fate to differentiate, self-renew, or maintain the stem niche (62-67) (**Figure 1.2**).

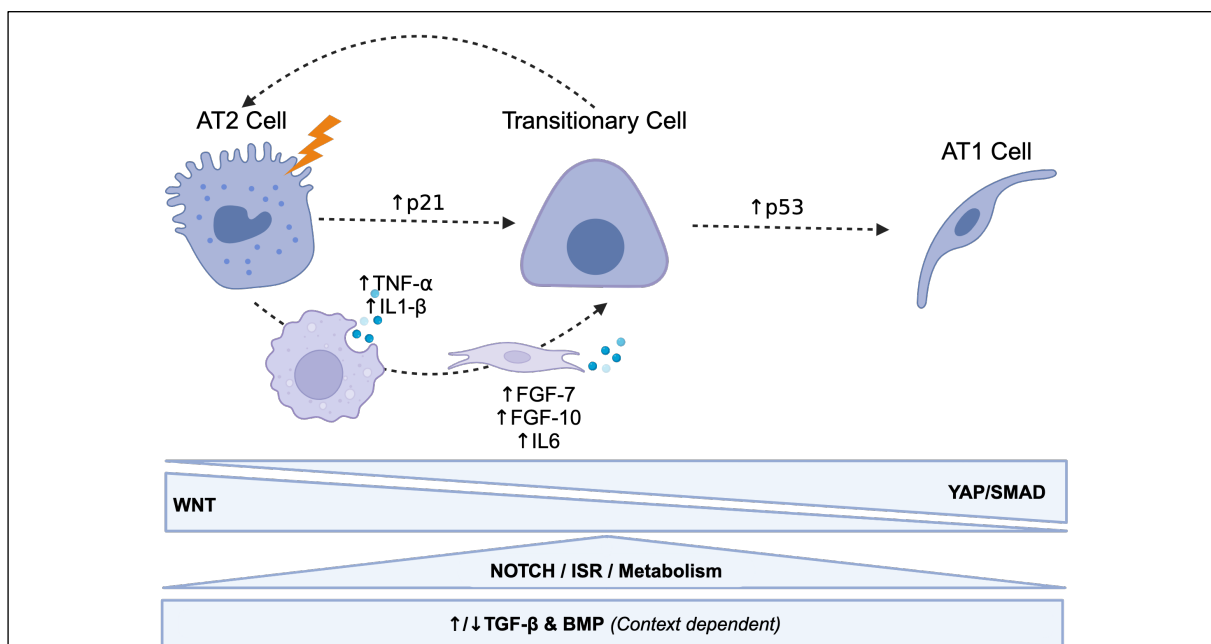


Figure 1.2: Molecular cues regulating AT2 cell self-renewal and differentiation

This figure illustrates the key interactions and signalling pathways involved in AT2 cell maintenance or differentiation into AT1 cells. WNT signalling plays a central role in preserving AT2 cells in a stem-like state, while attenuation of WNT and activation of YAP/SMAD signalling facilitates differentiation. Metabolic changes, stress responses, and NOTCH signalling are thought to transiently peak during the transitional phase. Secreted factors from alveolar macrophages and fibroblasts modulate AT2 cell fate - FGF7 supports AT2 identity, while IL-1 β and TNF α promote AT2 proliferation. These processes are tightly regulated by transient, context-dependent signalling dynamics.

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Alveolar repair is an energy-intensive process. Consequently, AT2 cells have a higher mitochondrial density than other lung cells, highlighting their dependence on mitochondrial function and energy metabolism (68). To meet these energy demands, AT2 cells alter several metabolic pathways (60, 61, 69). Aged AT2 cells often fail to make these metabolic adjustment, resulting in lower ATP production and diminished capacity for differentiation following injury (61). Furthermore, disruptions in metabolic pathways due to injury or lung pathologies can lead to aberrant repair mechanisms (69). Together suggesting disrupted mitochondrial metabolism and ATP production both negatively impact AT2 repair capacity.

In agreement, mitochondrial complex-I (NADH:ubiquinone oxidoreductase), the initial enzyme in the mitochondrial electron transport chain, is crucial for AT2-directed alveolar repair (70). A specific loss of complex-I in AT2 cells activates the mitochondrial integrated stress response (ISR). Analysis of multiple single-cell RNA-sequence datasets reveals that transitional cells display enriched ISR signatures (70). Interestingly, inhibiting the sustained ISR activation can improve repair outcomes when complex-I is lost in AT2 cells (70). This suggests that ISR activation may facilitate the transition of AT2 to AT1 cells and is subsequently switched off to allow for terminal differentiation.

Secreted factors from local cells also support the AT2 cell directed repair (71). Here we focus primarily on macrophage and fibroblasts derived cues. Fibroblasts predominantly interact through the AT2-fibroblast growth factor receptor 2 (FGFR2) pathway, where fibroblast-derived growth factors (FGF10 and FGF7) are essential for maintaining AT2 cell identity (55, 72). Loss of FGFR2 signalling in AT2 cells results in apoptosis and impaired healing post-injury (72, 73). Moreover, AT2-secreted transforming growth factor beta (TGF- β) or interleukin-1 beta (IL-1 β) stimulates fibroblasts to produce antagonists of the bone morphogenetic protein (BMP) signalling pathway, resulting in AT2 cell proliferation and differentiation (74). Macrophage derived-cytokines (IL-1 β , tumour necrosis factor alpha [TNF- α]) promote AT2 cell proliferation and differentiation (54, 58). Appropriate inflammatory signals are needed to ensure AT2-to-AT1 maturation, with unresolved IL1- β signalling leading to the accumulation of transitional cells (54).

Together this underscores the intricate regulation of alveolar repair, characterised by temporary precise modulation of signalling pathways. Disruption of these signals results in the accumulation of transitional cells, a hallmark observed in chronic lung diseases (54, 70, 75). The responsiveness and regulatory capacity of AT2 cells is contingent upon the nature of the injury and the cell's age, with a progressive loss of stemness observed in aging AT2 cells (61, 76).

1.1.4 Mitochondria in AT2 cells

Mitochondria are double-membraned organelles found in most eukaryotic cells, responsible for generating ATP through oxidative phosphorylation. Thought to have evolved from ancestral bacteria, they possess their own circular DNA and are central regulators of energy-dependent processes, particularly in energy demanding and high oxygen tissues such as the respiratory system (77, 78). A recurring theme in AT2 cell function is their reliance on mitochondria activity and metabolism to sustain their specialized roles.

AT2 cells are equipped with a relatively high mitochondrial density to meet their substantial energy demands (68). While glucose is the primary energy source for most mammalian cells, the lung maintains low intracellular glucose levels (79). AT2 cells appear to preferentially utilize fatty acid oxidation (FAO), likely due to the high lipid contents in the alveolar fluid. Impaired FAO results in AT2 cell apoptosis (80) and failed differentiation to AT1 cells (69). The energy source used by AT2 cells is likely context-dependent, varying with their functional state and nutrient availability.

AT2 cell-specific gene deletion murine models targeting mitochondrial dynamics (81) and respiration (70) result in spontaneous pulmonary fibrosis or respiratory failure, respectively. Furthermore, impaired mitochondrial metabolism increases susceptibility to experimentally induced fibrosis (69). These findings underscore the central role of mitochondria in maintaining AT2 cell health and implicate mitochondrial dysfunction as a key driver in AT2-mediated lung disease.

1.2 AT2 Cells in Lung Diseases

The dysfunction or loss of AT2 cells is central to lung diseases, including idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) (8). In disease, AT2 cells are thought to lose or alter their normal function, resulting in dysfunctional repair mechanisms, surfactant dysregulation, epithelial barrier breakdown and altered intercellular communication (**Figure 1.3**). Despite differing pathologies - with IPF being a restrictive lung disease and COPD an obstructive one - both involve significant AT2 cell damage and dysfunction, underscoring the importance of exploring AT2 cell biology and pathology to identify new therapeutic targets and improve treatments for lung diseases.

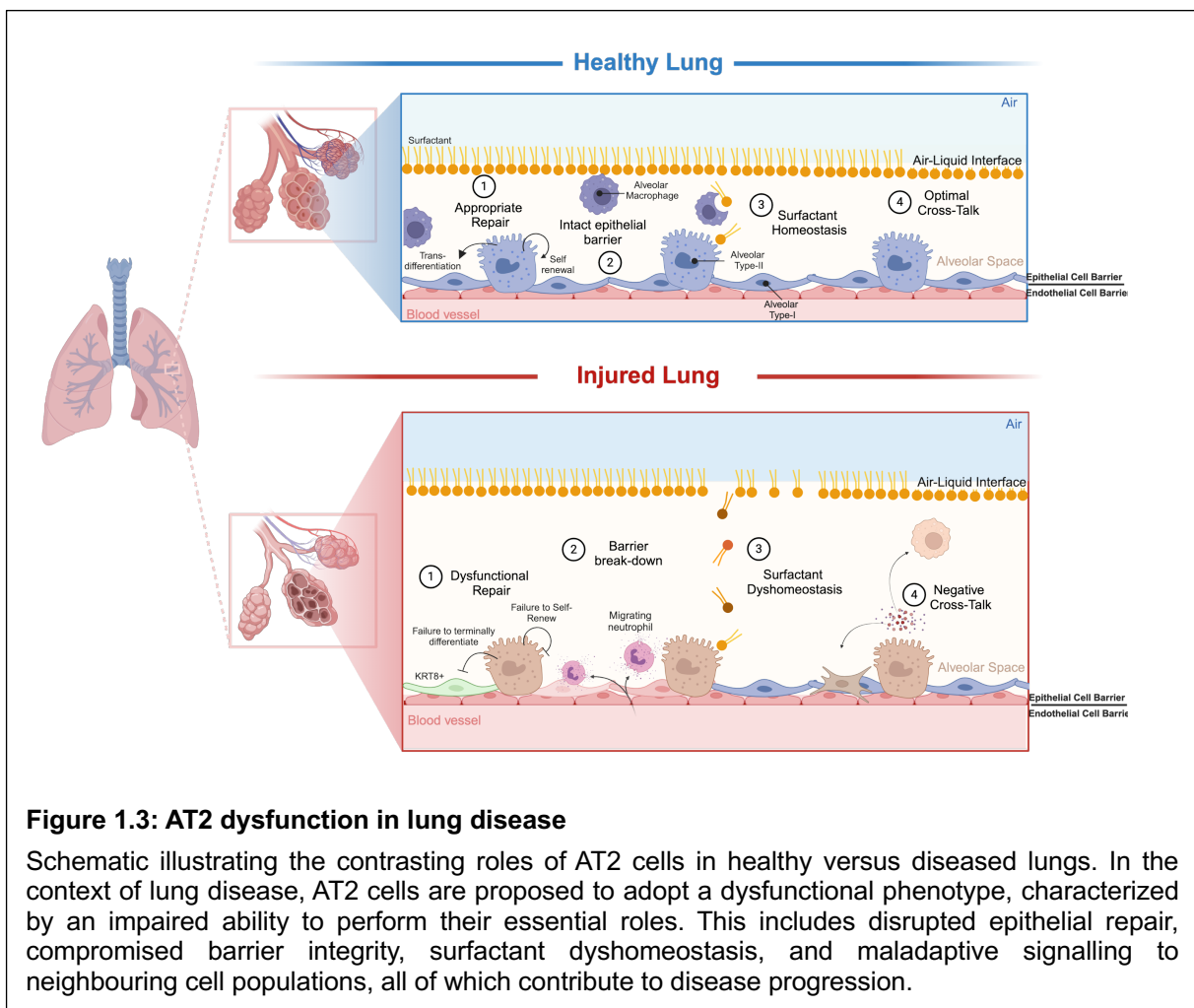


Figure 1.3: AT2 dysfunction in lung disease

Schematic illustrating the contrasting roles of AT2 cells in healthy versus diseased lungs. In the context of lung disease, AT2 cells are proposed to adopt a dysfunctional phenotype, characterized by an impaired ability to perform their essential roles. This includes disrupted epithelial repair, compromised barrier integrity, surfactant dyshomeostasis, and maladaptive signalling to neighbouring cell populations, all of which contribute to disease progression.

1.2.1 Idiopathic Pulmonary Fibrosis

IPF is a chronic, progressive interstitial lung disease (ILD) of unknown aetiology, characterised by irreversible fibrosis and a rapid decline in lung function (82, 83). IPF is a fatal disease with survival rates after diagnosis as low as 3 - 5 years (84). Current treatment options remain limited; with lung transplantation offering the most favourable outcomes for individuals with

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IPF. However, it is not a viable option for many due to factors such as age, comorbidities, and donor availability (84). Antifibrotic agents pirfenidone and nintedanib remain the sole approved pharmacological treatment option (85, 86). While these agents can slow disease progression, they do not reverse established fibrosis, underscoring the critical need to better understand disease mechanisms.

IPF pathogenesis involves complex interactions between genetic predisposition and repeated lung injury, resulting in aberrant fibrotic responses. The precise aetiology of IPF remains unclear, though both intrinsic factors (e.g., genetics, aging) and extrinsic factors (e.g., environmental exposures like cigarette smoke) have been implicated in disease progression (82). Genetic variants associated with alveolar stability, stem cell maintenance and cellular senescence; all of which directly impact AT2 cell specific functions, are among the strongest risk factors for IPF (87-89). Consequently, recent studies consider chronic/repeated alveolar epithelial injury as the central driver of aberrant repair and fibrotic remodelling in IPF.

1.2.2 Chronic Obstructive Pulmonary Disease

COPD is a progressive inflammatory lung disorder characterised by emphysema (alveolar destruction) and chronic bronchitis (bronchial inflammation). Together this results in persistent airway inflammation and obstruction, and structural remodelling of the small airways. Although COPD progresses gradually, it remains the third leading cause of death worldwide due to the high susceptibility of patients to acute exacerbations, often triggered by respiratory infections, leading to rapid and significant decline in lung function (90, 91). Similar to IPF, treatment options for COPD are limited, focusing primarily on symptom management, with no therapies currently available to halt or reverse disease progression (92). Together, this underscores the ongoing need for research into the molecular mechanisms underlying COPD susceptibility and pathogenesis.

Cigarette smoking is the primary risk factor for COPD; however, approximately 30% of individuals with COPD have never smoked, while many current-smokers do not develop the disease (93). This suggests a more complex and heterogeneous cause involving multiple risk factors beyond smoking. Indeed, genetic predisposition has also been implicated in COPD susceptibility (94). Emphysema, a principal pathological feature of COPD, arises from the irreversible destruction of alveolar structures and the inability to regenerate the alveolar epithelium after injury. It is posited that a loss of stem cell-like function in AT2 cells plays a pivotal role in this process.

1.2.3 The Casual Overlap of AT2 cell Dysfunction in IPF and COPD

In IPF the number of mature AT2 cells are reduced (95), likely due to cell death (96). Consistently targeted ablation of AT2 cells in murine models induces a fibrotic response (97, 98). However this fibrosis is transient and resolves over time, suggesting that while AT2 cell loss contributes to fibrogenesis, it is not the sole driver of the process. An additional explanation for AT2 cell loss is the accumulation of aberrant epithelial subsets in both IPF and murine models of fibrosis (75, 95). Notably, transitional cells co-expressing both AT1 and AT2 markers are observed, suggesting a failure of AT2 cells to transdifferentiate into AT1 cells or to self-renew.

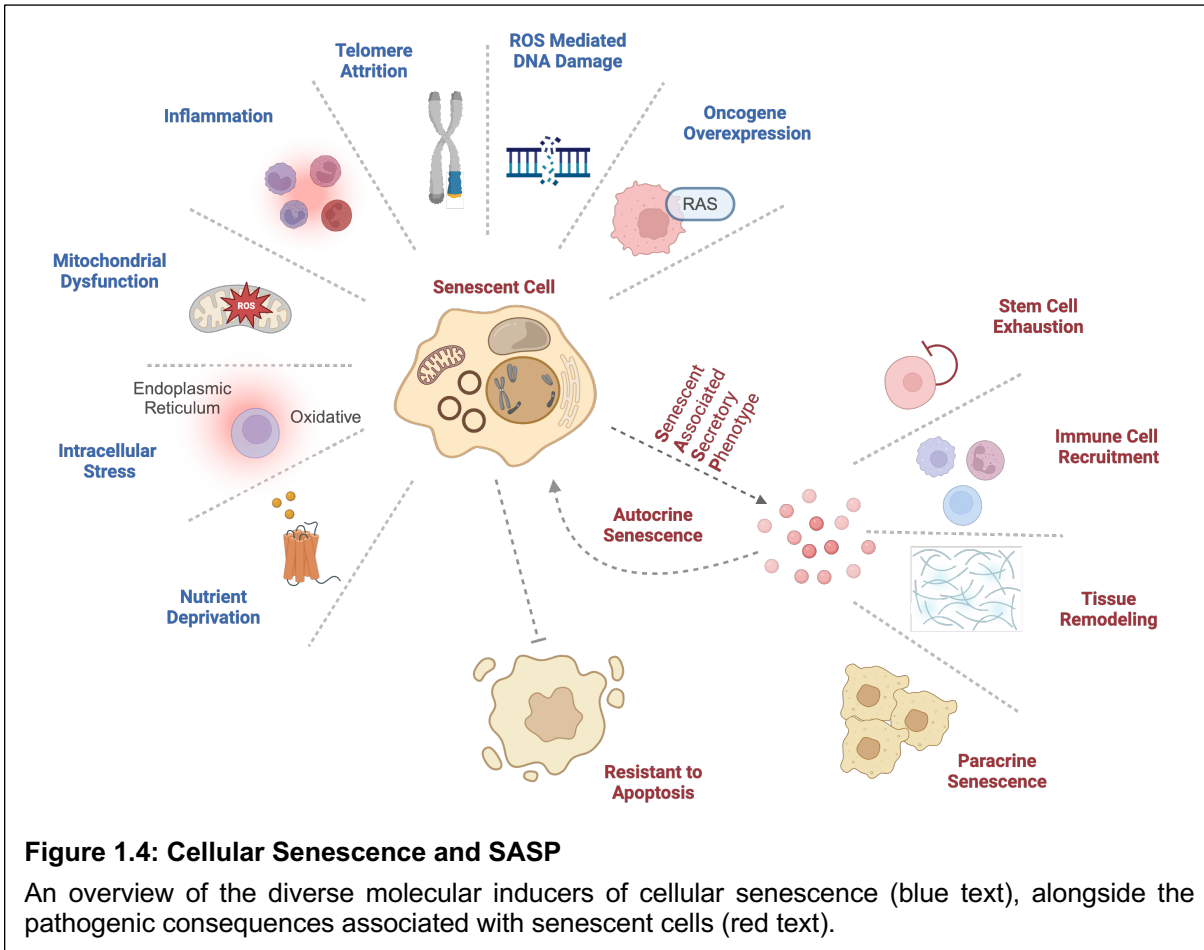
Similar to IPF, there is a depletion of healthy AT2 cells in COPD with an expansion of aberrant AT2 cell subsets (99, 100). In both human and murine models of COPD, these aberrant AT2 cells do not exhibit the transitional profiles described in ILD, but instead co-express markers of AT2 and club cells, suggesting an attempted yet impaired regenerative response (100). Supporting this, these cells fail to form organoids *ex vivo* (100). Additionally, a separate single cell RNA-sequencing (scRNA-seq) study in human COPD and mouse models reported a similar trend, showing loss of quiescent and WNT-active progenitor AT2 cells alongside enrichment of aberrant AT2 subsets (99). Notably, the absence or limited sample size of healthy smoker controls leaves it unclear whether these changes are disease-specific or smoke-induced.

In IPF, this arrested state in AT2 cells is characterised by a strong senescence-associated gene expression profile relative to healthy individuals (101). Similarly AT2 cells in COPD also show features of senescence, which correlates with disease severity in emphysema (102). Cellular senescence is a stress-response program, induced by a variety of intracellular and extracellular stressors (**Figure 1.4**) (103). Once activated, cells enter a state of 'permanent' cell-cycle arrest, regulated by cyclin-dependent kinase (CDK) inhibitors and develop a senescence-associated secretory phenotype (SASP) (103, 104). SASP is characterized by the release of pro-inflammatory cytokines, chemokines, proteases, growth factors and extracellular matrix components, which serve critical functions in promoting wound healing and repair by stimulating nearby cells and recruiting immune cells to clear damaged cells (105, 106). Additionally, secreted SASP factors can propagate senescence to neighbouring cells (107). While transient senescence is beneficial for tissue repair and regeneration, prolonged senescence and the excessive accumulation of senescent cells is detrimental and contributes

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to chronic inflammation and dysfunctional repair processes, as seen in IPF and COPD (**Figure 1.4**).

In both IPF and COPD, AT2 cells are characterised by the upregulation of cell-cycle arrest



proteins. Differentially, the SASP of AT2 cells in IPF adopts a more pro-fibrotic, pro-repair phenotype (101), whereas in COPD, AT2 cells are thought to exhibit a more pro-inflammatory SASP (99).

The senescence of AT2 cells in disease is likely multifactorial. Notably, senescence is linked with aging, and both COPD and IPF predominantly affect older individuals. Genetic factors further support this association, with polymorphisms in TERT and TERC linked to both familial and sporadic IPF (87-89) and rare variants in TERT associated with emphysema (94).

Beyond aging, chronic injury drives COPD and IPF pathogenesis by increasing oxidative stress and ROS production in the lung (108, 109). Several antioxidants are present within the cell to manage and counteract the production of ROS (110). However, if ROS accumulates, it can catalyse DNA and cellular damage, promoting senescence and further dysfunction (110).

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Mitochondria are one of the main sources of cellular ROS (110), generating it as a by-product of cellular respiration. Furthermore, mitochondrial dysfunction independently contributes to cellular senescence (103). Given the critical role of mitochondria in both senescence and ROS production, any dysfunction or damage to these organelles can significantly impact AT2 cell health. Indeed, mitochondrial abnormalities in AT2 cells have been implicated in both COPD and IPF, as detailed in **Table 1.1**,

Specifically, AT2 cells in both IPF and COPD exhibit altered mitochondrial bioenergetics. In IPF, there is an overall decrease in metabolism, including reduced oxidative phosphorylation (OXPHOS) (111), FAO (69) and glutamine metabolism (60); likely reflecting diminished AT2 cell health. Conversely, AT2 cells from cigarette smoke (CS)-exposed mice demonstrate increased FAO, which may represent an adaptive response to oxidative stress induced by CS (112).

Furthermore, disruptions in mitochondrial dynamics are a common feature across both diseases. AT2 cells isolated from emphysematous lungs show impaired mitochondrial fission and fusion (109). Similarly, AT2 cells from IPF donor lungs display increased mitochondrial mass, suggesting abnormalities in these processes (113). Notably, a specific loss of mitofusin 1 and 2, genes that regulate mitochondrial fusion, leads to spontaneous lung injury and fibrosis (81). Together, underscoring the critical role of AT2 mitochondrial integrity in the pathogenesis of both COPD and IPF.

Table 1.1: AT2-mitochondrial dysfunction in IPF and COPD

Mitochondrial Process	Process Name	Changes in Mitochondrial Processes in AT2 cells	
		IPF	COPD
Bioenergetics	Aerobic/ Anaerobic glycolysis	↓ Glutamine metabolism in AT2 cells in IPF and murine BLM-induced fibrosis (60)	n/a
	FAO	↓ FAO in AT2 cells from IPF donors (69)	↑ FAO in AT2 cells from smoked mice (112)
	OXPHOS	↓ OXPHOS in AT2 cells from IPF donor (111)	n/a

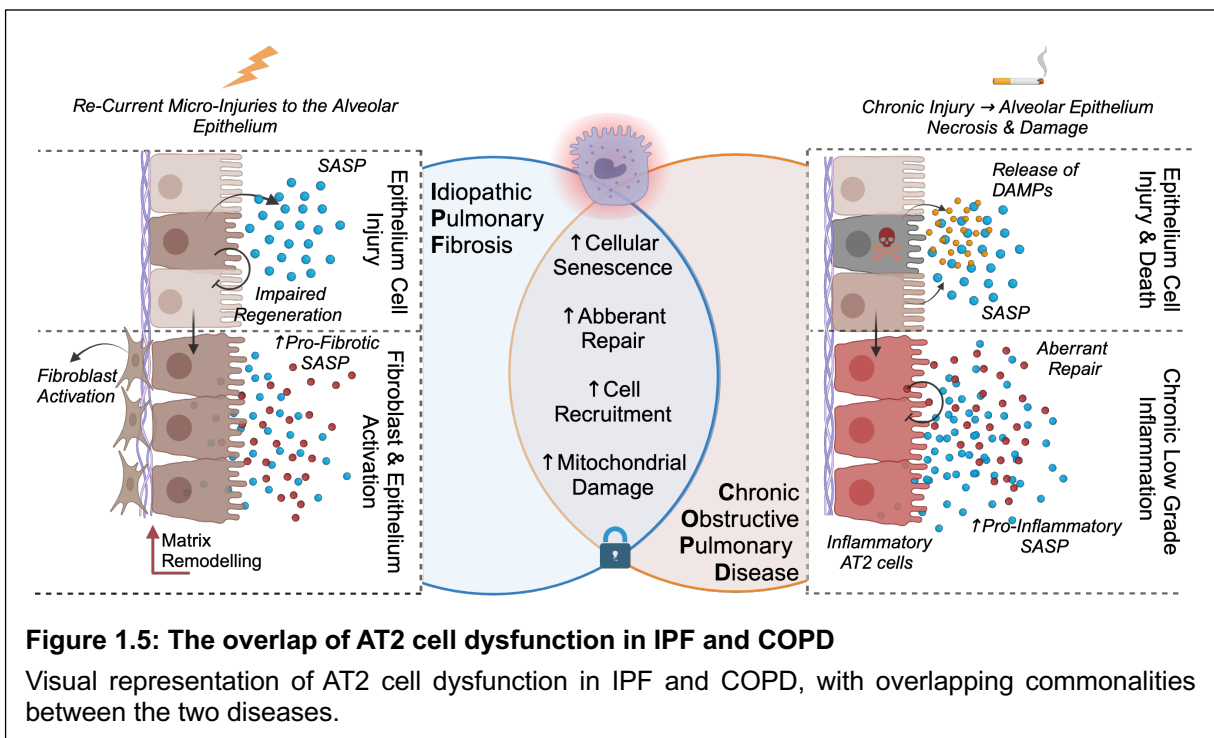
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Mitochondrial Process	Process Name	Changes in Mitochondrial Processes in AT2 cells	
		IPF	COPD
	TCA	↓ Complex-I and Complex-IV activity and ATP levels in IPF (whole lung) (113)	n/a
mtDAMPS	mtDNA	↑ Mitochondria Mass in AT2 cells from IPF donor (may indicate ↑mtDNA) (113)	↓ mtDNA copy number in AT2 cells individuals with emphysema (109)
	mtROS	↑mtROS in IPF (whole lung) (108)	↑ mtROS in isolated AT2 cells from emphysema (109)
Dynamics	Mitophagy	↓ Mitophagy in AT2 cells of individuals with IPF (113)	↑ Mitophagy in AT2 from emphysema (109)
	Biogenesis	n/a	↓ Biogenesis in AT2 cells from emphysema lungs(109)
	Fission/ Fusion	Mitochondrial swelling of AT2 cells in IPF (113) Spontaneous fibrosis in mice harbouring AT2-depletion of Mitofusin 1/2 (81)	Impaired fission/fusion in AT2 cells from emphysema lungs (109)

From the above, it is evident that there is significant overlap in AT2 cell dysfunction in IPF and COPD (**Figure 1.5**). In these diseases, AT2 cells fail to repair the epithelium with their SASP promoting a pro-fibrotic (IPF) or pro-inflammatory (COPD) microenvironment, orchestrating aberrant responses in surrounding cells. The critical question still remains: 'What mechanisms are responsible for the persistent senescence and dysfunction of AT2 cells in chronic lung diseases?'

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Previous studies have identified iron overload as a prominent feature in IPF, with similar findings observed in individuals with COPD (114-117). Notably, mitochondrial iron accumulation has been recognized as a critical element in the pathogenesis of COPD (118). Furthermore, iron accumulation in the lungs has also been implicated in the initiation and progression of cellular senescence and SASP (119). Considering the pivotal role of mitochondrial dysfunction within AT2 cells, coupled with elevated ROS and SASP/senescence in both diseases, a notable gap in the literature emerges raising a critical question: '**How does MITOIRON contribute to AT2 cell pathology in COPD and IPF?**'



1.3 Overview of Systemic Iron Uptake and Regulation

Iron is an essential transition metal utilized by almost all species, with approximately 2% of human genes encoding an iron-protein (iron-binding, heme binding and iron sulfur proteins = ironome) (120). Given iron's oxidative/reductive characteristics, which allow it to accept and donate electrons, it is indispensable for several key biological processes such as oxygen transport, cellular respiration, enzymatic functions, and nucleic acid synthesis. However, this characteristic acts as a double edged sword as iron can also catalyse the generation of free radicals via Fenton chemistry (121). Free radicals are unstable and can cause damage, dysfunction and ultimately cell death. As a result, iron uptake, storage, transport and utilization in every cell is tightly regulated.

In mammals, iron is sourced exogenously through the diet (~1mg of iron) and endogenously via recycling and degradation of iron-containing molecules. Dietary iron absorption occurs mainly in the duodenum and exists in two forms: heme and non-heme iron (122). Non-heme iron, which exists predominantly as ferric (Fe^{3+}), must be reduced to ferrous iron (Fe^{2+}) by the enzyme ferrireductase duodenal cytochrome B (DCYTB) to facilitate its transport into the cytosol via divalent metal transporter 1 (DMT1) (122, 123). Heme-iron is absorbed through heme carrier protein 1 (HCP1) (124) and exported via transmembrane heme-exporters feline leukemia virus subgroup c receptor (FLVCR) (125, 126). Alternatively, heme-iron can be reduced by heme oxygenase (HO) activity which cleaves heme-iron complexes, releasing iron for absorption (126).

The main source of circulatory iron comes from the recycling of senescent red blood cells (RBC) via macrophages in a process called erythrophagocytosis (127). The contents of the phagocytosed RBC are then shuttled into the cytosol of the macrophage, where the iron is either stored, recycled internally or exported into the bloodstream. HO1 plays a pivotal role in the recycling of iron, with mice lacking HO1 developing anaemia, significantly low serum iron levels, iron accumulation in the liver and spleen and chronic inflammation (128). Macrophage dependent recycling is an essential biological function that plays a key role in iron homeostasis, highlighting the cross-link between immunity and iron homeostasis in biology.

Plasma iron is subsequently bound to carrier proteins, mainly transferrin (TF), to allow for iron delivery to all cell types throughout the body. The transferrin receptor (TFR) on plasma membranes of cells is a glycoprotein containing two high affinity binding sites for Fe^{3+} , allowing it to bind one or two atoms of iron, forming mono-ferric and di-ferric conjugates respectively

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(129). TFR loss or deficiency results in tissue iron overload and anaemia, highlighting the key role of TFR in systemic iron handling and distribution (130).

Systemic iron homeostasis is centred around the liver and the hepcidin-ferroportin regulatory axis (131). Hepcidin (encoded by *HAMP*) is expressed by hepatocytes in the liver (131). Hepcidin levels are regulated by high systemic iron conditions and inflammatory signals, including IL-6. When induced, hepcidin then regulates iron levels by inhibiting the absorption of dietary iron and the release of iron from hepatocytes and tissue macrophages through the degradation of the sole known iron-ion exporter ferroportin (FPN) (132). Once optimal iron levels are restored, hepcidin expression is normalized and plasma iron levels are increased respectively. The latter is true for lower systemic iron levels, in which hepcidin expression is negatively regulated, promoting the increase of iron absorption (132).

Approximately 75% of total body iron is utilized by red blood cells for erythropoiesis and incorporation into hemoglobin, which is essential for oxygen transport throughout the body (133). The remaining iron is distributed across all tissues, where it supports vital cellular functions. While iron and heme are traditionally linked to hemoglobin in red blood cells, they are indispensable across all cell types, where they support fundamental processes such as mitochondrial respiration, DNA synthesis, and metabolic regulation. Despite its universal necessity, the specific roles of iron in supporting specialized cellular functions remain underexplored and often underappreciated.

1.3.1 Cellular Iron Absorption and Uptake

Each cell in the body is equipped with specialized machinery for the uptake (1), utilization (2), storage (3), and export (4) of iron (**Figure 1.6**); with specific cells including hepatocytes, macrophages, enterocytes and erythroid progenitor cells having a higher affinity for iron.

Most cells absorb iron through receptor mediated endocytosis of the transferrin receptor (TFR1/CD71) which is ubiquitously expressed. TF-Fe³⁺ binds CD71 which is then subsequently endocytosed allowing for the release of Fe³⁺ (134). Once Fe³⁺ is released, CD71 and TF are both recycled back into the circulation allowing for the uptake of more iron (134). Fe³⁺ is then reduced to its ferrous form via the ferric reductase six transmembrane epithelial antigen of prostate 3 (STEAP3), allowing Fe²⁺ to be transported from the endosomal membrane into the cytosol via DMT1 (135). TFR2 can also facilitate the import of transferrin bound iron (TBI) in erythrocytes, but whether the same is true for specialised cells remains unknown (136).

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Aside from TFR- Fe^{3+} acquisition cells can acquire non-transferrin bound iron (NTBI) via DMT1, zinc transporter ZIP8 (SLC39A8) and ZIP14 (SLC39A14) (137, 138). Protein-bound iron in the form of ferritin, classically known for its iron storage capacity (discussed below), and lipocalin 2 (LCN2) bound iron, an iron siderophore, can also be acquired via receptor mediated endocytosis (139-141). Recently, CD44 has also been proposed to facilitate the endocytosis of iron bound to hyaluronate, a glycosaminoglycan found in the extracellular space (142). Heme uptake is facilitated by HRG1 and SLCO2B1 (143). During hemolysis, heme and hemoglobin released into the plasma are bound by scavengers including haptoglobin and hemopexin and are removed from circulation by receptors on macrophages and hepatocytes (143). Of note, many of these NTBI and heme uptake pathways are cell-specific and context-dependent, frequently being identified under conditions where the classical TBI uptake is impaired or in response to cellular stress.

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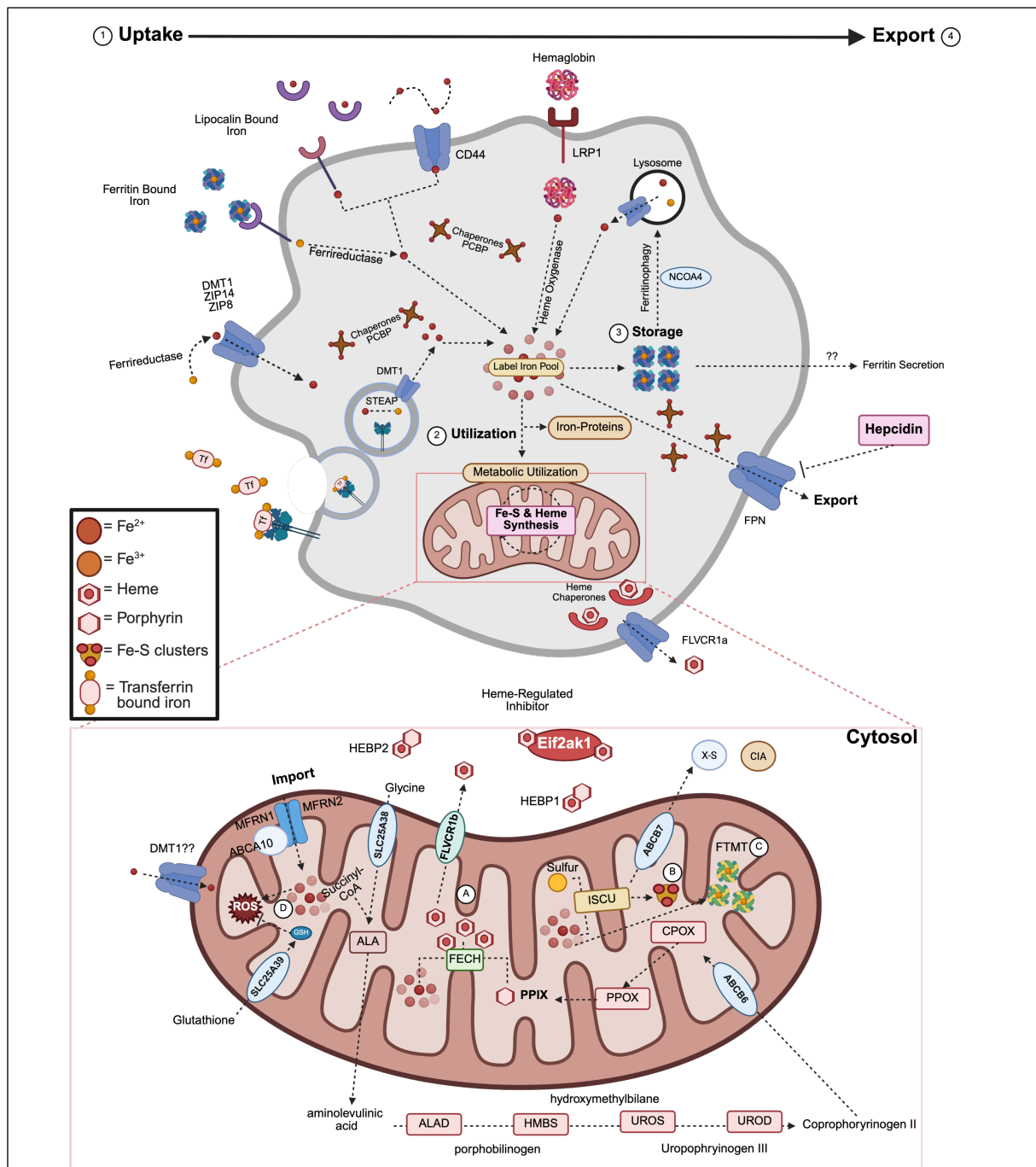


Figure 1.6: Cellular and Mitochondrial Iron Regulation

(1) Uptake: The majority of cellular iron is acquired as transferrin-bound iron (TBI), which binds to the transferrin receptor and is internalized via endocytosis. Within endosomes, Fe^{3+} is released, reduced to Fe^{2+} , and transported into the cytosol by DMT1. Non-transferrin-bound iron (NTBI) can also enter cells via ZIP8, ZIP14, or DMT1. In certain cell types, iron may be internalized as heme-containing complexes or hyaluronate-bound iron. (2) Utilization: Once in the cytosol, iron is predominantly trafficked to mitochondria for use in (a) heme biosynthesis, (b) iron-sulphur (Fe-S) cluster formation, (c) storage and (d) mitochondrial labile iron pool. Cytosolic iron chaperones, such as PCBP1 and PCBP2, facilitate the targeted delivery of iron to subcellular compartments and client proteins. (3) Storage: Surplus iron is safely stored in ferritin nanocages to prevent oxidative damage. If needed, stored iron can be mobilised via ferritinophagy, a selective autophagic process mediated by NCOA4. (4) Export: Iron is exported from the cell through ferroportin, the only known iron exporter. Additionally, FLVCR1 enables the export of heme. Ferritin endocytosis may contribute to iron export.

1.3.2 Mitochondria and Iron Utilization

Once in the cytosol, iron can be utilized as a cofactor for enzymes such as prolyl hydroxylases (PHD) (described below), however, iron is predominantly trafficked to mitochondria for downstream utilization.

Cytosolic iron must be transported across both the highly permeable outer mitochondrial membrane (OMM) and the tight diffusion barriers of the inner mitochondrial membrane (IMM) (144, 145). The precise mechanism of iron delivery to mitochondria remains unclear. However, it is hypothesized that TBI reaches the OMM through transferrin-containing endosomes, in a process termed 'kiss and run' (146). Recent studies have identified DMT1 on the OMM suggesting NTBI can also be transported into the mitochondria (147).

Transport across the IMM is facilitated by two members of the mitochondrial solute carrier family, Mitoferrin 1 (MFRN1/SLC25A37) and Mitoferrin 2 (MFRN2/SLC25A28). ATP-binding cassette 10 (ABCB10) complexes with MFRN1 to regulate mitochondrial iron transport in erythroblasts (148). Homologues of these Mitoferrins (MFRNs), *MRS3* and *MRS4*, were first described in *S.cerevisiae* yeast (149-151).

MFRN1 has been characterised as playing an important role in erythroid cell development, with its deletion in mice and zebrafish resulting in hypochromic anaemia and erythroid maturation arrest (152). MFRN1 also interacts and binds to several mitochondrial proteins that have functional roles in heme-biosynthesis (148, 153). Recent advancements in single-cell sequencing have revealed broader expression of MFRN1 across various cell types, suggesting a wider role in cellular iron metabolism (154). The precise role of MFRN2 on the other hand is largely unknown, however, studies have suggested a role for MFRN2-mediated mitochondrial iron import in other non-erythroid cell types as it is widely expressed in mammalian tissues (155-157).

A loss or decreased expression of MFRN1 and/or MFRN2 results in decreased mitochondrial iron content, reduced Fe-S and heme-synthesis (156, 158). In yeast, loss of both *MRS3/4* results in impaired growth in low iron media conditions (149-151). Similarly, specific loss of MFRN1/2 in hepatocytes significantly reduces proliferation and repair, which is exacerbated under low-iron conditions (158). This suggests that MFRN1/2 are high-affinity mitochondrial iron transporters and in conditions of iron abundance, alternative mitochondrial iron importers may compensate. There is evidence for mitochondrial calcium uniporter protein (MCU) as an

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alternative mitochondrial iron transporter under iron-limiting conditions, though further investigations are needed (159).

Once imported into mitochondria, iron is primarily utilized for (a) heme and (b) Fe-S cluster synthesis as described in detail below. Alternatively, iron can also be stored as mitochondrial ferritin (MTFT) (c) or reside in the mitochondrial free labile iron pool (LIP) (d) (**Figure 1.6**).

(a) Heme Biosynthesis

Heme is a complex of ferrous iron and tetrapyrrole ring of heme (Fe-PPIX). Heme synthesis is a highly conserved biochemical pathway that involves multiple steps across cellular compartments. The first step and the final two steps occur in the mitochondria, while the remaining six intermediate enzymatic steps take place both in the mitochondrion and the cytosol (**Figure 1.6**). Heme synthesis first step is governed by the rate limiting enzyme 5-aminolevulinate (ALA) synthase, which catalyses the formation of ALA from glycine and succinyl CoA (160). The ALA product is subsequently converted to porphobilinogen (PBG) by ALA dehydratase, with PBG molecules then polymerized into hydroxymethylbilane (HMB) by PBG deaminase (160). HMB is subsequently converted to uroporphyrinogen III, which is decarboxylated to coproporphyrinogen III by uroporphyrinogen decarboxylase (UROD) (160). This compound is oxidized to protoporphyrinogen IX, and finally, protoporphyrinogen IX is oxidized to form protoporphyrin IX (PPIX) (**Figure 1.6**) (160). Finally ferro-chelatase (FECH), an iron-sulfur (Fe-S) containing protein, inserts ferrous iron into PPIX forming heme (161).

Heme serves as a crucial prosthetic group for oxygen transport proteins like hemoglobin and myoglobin, as well as various oxidoreductases such as cytochrome c and cytochrome p450 catalysing both oxidative and reductive reactions essential for oxygen transport, electron transfer and enzymatic activities (162). Additionally, heme plays a significant role in miRNA processing, gene regulation, ion channel activities, and cellular functions like adipogenesis and inflammation, underlining its broad influence on metabolic processes (160). Prosthetic heme can catalyse both oxidative and reductive reactions and plays an essential role in oxygen transport and storage, electron transfer, enzymatic reactions, signal transduction, ligand binding and control of gene expression. Once synthesized, heme is either utilized locally in the mitochondrion or exported via FLVCR1b for use in the forementioned functions (125). Once exported, binding proteins including progesterone receptor membrane component 2 (PGRMC2) and heme binding proteins (HEBP) facilitate heme transport throughout the cell (163).

(b) Fe-S Biogenesis

Fe-S clusters are ubiquitous co-factors found across all clades of biology. Versatile in shape, Fe-S can present with either a ferric or ferrous iron atom, allowing them to participate in redox reactions. These inorganic clusters are essential in biology and support several cellular processes including energy production, DNA maintenance, protein translation, lipid synthesis, regulation of gene expression and many more (164). Conserved across eukaryotes, there are 18 known mitochondrial Fe-S cluster assembly (ISC) proteins and 11 known cytosolic Fe-S protein assembly co-factors (CIA) that play regulatory roles in the *de novo* synthesis of Fe-S clusters (165).

Similarly to heme synthesis, Fe-S biogenesis occurs across the mitochondrion and the cytoplasm. Beginning in mitochondria, specific proteins synthesize [2Fe-2S] clusters on scaffold proteins using iron, cysteine, and electrons. These clusters are then transferred and sometimes converted into [4Fe-4S] clusters through a sophisticated network of carrier proteins and chaperones. [4Fe-4S] clusters are subsequently inserted into their target proteins. Locally in mitochondria, Fe-S clusters are inserted into aconitase (ACO2), complex I (NADH:ubiquinone oxidoreductase), complex II (Succinate dehydrogenase), lipoic acid synthase and FECH. Fe-S cluster biogenesis is a complex multistep process, readers are referred elsewhere for a more detailed review (164, 165).

Mitochondria orchestrate a multitude of essential cellular processes to meet the energy demands of metabolically active cells. Studies indicate that mitochondria, depending on the cell type, may harbour up to twice the concentration of iron compared to the cytosol (166), underscoring their critical role in iron metabolism. Mitochondrial metabolism is also highly iron-dependent; with key enzymes ACO2 and SDH incorporating Fe-S to carry out their catalytic functions in the tricarboxylic acid (TCA) cycle and in oxidative phosphorylation facilitating electron transport (167).

Co-factors produced in the TCA cycle transfer electrons to the electron transport chain (ETC), where they pass through four main complexes - Complex-I (NADH dehydrogenase, containing Fe-S clusters), Complex-II (succinate-ubiquinone oxidoreductase, containing both Fe-S clusters and heme), Complex-III (cytochrome bc1 complex, containing Fe-S clusters) and Complex-IV (cytochrome c oxidase, containing heme groups) - that utilize iron-based co-factors to facilitate electron transfer, culminating in the synthesis of adenosine triphosphate (ATP) (162). ATP serves as the universal energy currency for all cellular activities, especially critical in energy-intensive cells, including AT2 cells.

Additionally, electron transfer flavoprotein dehydrogenase (ETFDH) enzymes also contain Fe-S clusters play pivotal roles in transferring electrons from FAO processes to the ETC (162), which may be of particular relevance to AT2 cells given their high dependence on FAO-based metabolism (69). The intricate overlap between mitochondrial metabolism and MITOIRON underscores how fluctuations in either can cascade into significant disturbances in cellular function and health.

1.3.3 Iron Storage and Export

Free iron, if not utilized, is stored in a “ferritin-nanocage” which consists of a combination of ferritin heavy and ferritin light chains (168). The ferritin complex stores iron in its stable Fe^{3+} state in cells until it is required by the cell (169). During low intracellular iron level conditions, nuclear receptor coactivator 4 (NCOA4) is upregulated. NCOA4 expression mediates the release of iron from ferritin via ferritinophagy (170). In the cell, iron can be transported by poly(rC)-binding protein 2 (PCBP2) which chaperones to the basolateral side of the cell, where it is exported into the circulation by FPN (171).

Subsequently, iron can be exported as heme-iron or ferritin secretion in exosomes. Heme iron is primarily exported via transmembrane heme-exporters (FLVCR) (125). Additionally, under heme limiting conditions ATP-binding cassette subfamily (MRP-5) facilitates the transport of heme out of intestinal cells into circulation (172). Iron-loaded ferritin is secreted via non-classical secretory pathways; through secretory autophagosomes and multivesicular bodies (MVBs) leading to exosome release (173).

1.3.4 Iron Regulation

In a healthy organism, iron levels must be maintained at the optimal level while also adjusting to the iron requirements of each individual. Sophisticated mechanisms have been developed where iron levels can be sensed and subsequent absorption, utilization or recycling can take place in order to maintain homeostasis.

At the cellular level, iron is largely regulated by iron regulatory protein 1 (IRP1) and iron regulatory protein 2 (IRP2). Once activated, IRPs bind to iron responsive elements (IREs) within the untranslated regions of mRNAs associated with proteins involved in cellular iron homeostasis in attempt to restore and regulate iron levels (143). Primary iron gene targets of the IRP1/2 system include; *FTL*, *FTH1* (storage), *TFR* (uptake) and *SLC40A1* (export).

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Additionally the IRP/IRE axis can indirectly affect ISC proteins (Fe-synthesis), ALAS (heme-biosynthesis) and other iron uptake pathways.

Under normal iron conditions, cytosolic iron-sulfur assembly component 2A (CIAO2A) facilitates the transfer of a [4Fe–4S] cluster to IRP1, converting it into a functional aconitase (143). However, in iron-deficient or oxidative conditions, IRP1 loses this cluster and its aconitase activity, shifting to a structure that binds IREs, including TFRC to facilitate cellular iron uptake. IRP2 does not have aconitase activity and is regulated by FBXL5, which mediates its degradation in response to iron and oxygen levels through its hemerythrin-like and leucine-rich repeat domains (143). Interestingly, IRE structures have been identified in mRNAs encoding cell-cycle and metabolic proteins, underscoring the iron-dependent regulatory mechanisms embedded in cellular proliferation and survival (174, 175).

Iron regulation can also be mediated through hypoxia inducible factors (HIF) axis (176) or through heme regulated inhibitor (HRI/ EIF2AK1) stress response. HIF proteins play a crucial role in the cellular response to oxygen levels. In oxygen replete conditions, PHD enzymes hydroxylate HIF- α subunits, targeting them for VHL-mediated degradation. PHDs are 2-oxoglutarate dependent dioxygenases that require Fe^{2+} as an essential cofactor for catalytic activity. As a result iron deplete conditions prevent PHDs from degrading HIF-subunits resulting in their stabilization and activation of hypoxia-responsive genes (176). Subsequently, activated HIF subunits can then directly regulate the gene expression of IREs to prompt iron uptake in iron deplete conditions (177).

The heme response inhibitor (HRI) is one of four kinases involved in the ISR. Under normal conditions, HRI is bound by heme molecules, maintaining the kinase in an inactive state. Under heme or iron deficiency, HRI is activated and phosphorylates the α -subunit of eukaryotic initiation factor 2 (eIF2 α); thereby inhibiting global protein synthesis (178). Historically, the role of HRI was predominantly associated with erythroid heme sensing, aligning globin translation with heme availability in iron-deficient conditions. However, recent studies indicate that HRI can also be activated independently of heme during mitochondrial dysfunction (179, 180) and may contribute to innate immune signalling (181). Despite these findings, the precise role of HRI in heme and iron sensing in non-erythroid specialized cells remains poorly defined and warrants further investigation.

1.4 Iron Regulation in the Lung

The lung is essential for gas exchange and hosts a diverse array of cell types critical for pulmonary defence and homeostasis. Uniquely, the lung is continuously exposed to both endogenous iron from the pulmonary vasculature and exogenous iron from inhaled particles. Consequently, the regulation and use of iron in the lung is emerging as an important area in pulmonary research, with iron dysregulation increasingly implicated in the pathogenesis of both acute and chronic lung diseases (182).

Iron in the lung is primarily acquired through the pulmonary vasculature in the form of TBI, NTBI, cell-free Hb/heme or lactoferrin-bound iron (182). Additionally, iron can be acquired from the airspace, where iron carrier proteins including TFR, lactoferrin, ferritin, ceruloplasmin and LCN2 bind iron to prevent damage and facilitate uptake by epithelial cells (183-185). The AE can actively secrete LCN2, an iron-binding glycoprotein that sequesters iron to limit microbial growth, underscoring its dual role in innate immunity and iron regulation (182). Whether or not AT2 cells also secrete LCN2 remains to be elucidated, however, single cell data sets highlight a subset of AT2 cells that increase *Lcn2* expression after injury, suggesting they may harness this function (75). Intratracheal instillation of free iron or targeted ablation of endothelial cells, leading to vascular haemorrhage, can overwhelm these protective iron carrier proteins, resulting in significant lung damage (119, 184, 185). Additionally, heme-derived iron compounds in the alveolar space also compromise lung health by increasing alveolar permeability and disrupting epithelial cell barrier function, both *in vitro* and *in vivo* (186, 187). Together, this underscores the importance of maintaining appropriate iron levels in the lung. Evidence from systemic iron dysregulation studies suggests that the IRP/IRE axis is predominantly responsible for cellular iron regulation in the lung (188, 189).

Cellular iron uptake receptors including TFR1, ZIP14 and DMT1/DCYTB have been described in the lung (182, 190, 191). The uptake of iron through non-classical pathways in lung cells, such as TFR2, CD44-mediated hyaluronate-iron, LCN2-iron or ferritin-iron described above remains unclear. Systemic iron overload in TFR2-mutant mice leads to significant iron deposition in the lung, particularly in the airway basement membrane, blood vessels and tissue macrophages (115). The presence of LCN2 and ferritin in bronchoalveolar fluid (BALF) suggests potential mechanisms for iron absorption. AT2 cells have demonstrated the capacity to internalize native ferritin (31). The uptake of iron-bound ferritin by AT2 cells and whether other lung cell types utilize similar endocytic mechanisms, remains to be determined. Surplus iron in lung cells is either sequestered within ferritin nanocages (as described above) or exported via FPN (192, 193). FPN expression is regulated by locally or systemically

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produced hepcidin (188, 189, 194). Point mutations in FPN result in iron accumulation in the alveolar space and restrictive lung disease (189), thickening of the air-blood barrier and AT2 cell hypotrophy and hyperplasia (188). Hepatocyte-specific hepcidin knock-out mice exhibit the same pulmonary iron overload seen in global hepcidin deficiency, indicating that circulating hepcidin is the primary regulator of lung iron homeostasis (195). Airway-derived HAMP appears to be linked to innate immune responses with local silencing of airway HAMP reducing AM iron content and impairing their phagocytic function (194). Consistently, *Hamp*^{-/-} AMs exhibit reduced IL-6 and TNF- α production upon *S. pneumoniae* infection (196). Together this would suggest that the alveolar region and alveolar associated cells are particularly susceptible to disruptions in the hepcidin/ferroportin regulatory loop, relying on iron homeostasis to carry out their normal functions, including alveolar repair (AT2 cells) and phagocytosis (AMs).

Furthermore, the HIF–iron axis as described above, tightly links iron homeostasis to oxygen sensing, which given the lungs high oxygen content is particularly relevant to cells at the air-blood barrier (176). This interplay is proposed to serve as an additional regulatory mechanism in pulmonary iron homeostasis (182).

1.4.1 Iron and Lung Disease

In the last ten years, dysregulated iron homeostasis has emerged as a significant research interest, with it increasingly recognized as a contributing factor in lung diseases (182). In IPF and COPD, studies have demonstrated a significant increase in iron availability within the BALF, where elevated iron levels correlate negatively with disease severity (114-117). In IPF, these increased iron levels are thought to exacerbate alveolar injury and fibrosis by enhancing oxidative stress through increased ROS production (116), with the same association likely true for COPD. In COPD, iron deposition in the lung is associated with an increased risk of infection-based exacerbations (197).

Genetic research suggests a potential role for iron in increasing susceptibility to respiratory diseases. Genome-wide association studies (GWAS) have identified the *IREB2* gene, which encodes the iron regulatory protein IRP2, as a susceptibility gene for COPD (198). Moreover, polymorphisms in *IREB2* are associated with COPD in individuals with *SERPINA1* mutations (causative of alpha-1 antitrypsin deficiency) (199). Interestingly, mutations in the hemochromatosis (*HFE*) gene, known to cause iron overload, strongly correlate with alpha-1 antitrypsin deficiency (200). Additionally, a higher frequency of *HFE* polymorphisms has been

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observed in individuals with IPF (201). Exposure to exogenous iron has been linked to increased susceptibility to respiratory diseases; for instance, individuals working in iron ore mines often develop persistent respiratory symptoms that continue even after exposure ends (202). Together this would suggest that iron may play a significant causative role in the development of lung diseases.

Considering the cellular implications of increased iron levels in the lung, AMs were identified as the predominant iron-positive cell in both IPF (203, 204) and COPD (205, 206). In IPF, AMs lacking TFR1 expression were more abundant and associated with decreased survival (207). Similarly, in COPD, iron-laden macrophages correlated negatively with disease severity (205, 206) and had decreased phagocytic capacity (197). These findings indicate that iron accumulation in lung disease disrupts AM phagocytic function, potentially enhancing the lung's vulnerability to opportunistic pathogens that exploit excess iron for growth.

Emerging evidence also suggests that dysregulated lung-iron metabolism may negatively impact alveolar epithelium, particularly AT2 cells. Cheng *et al.* (2021) suggest iron deposition in AT2 cells drives iron-dependent cell death pathways, contributing to BLM-induced fibrosis (208). However, measurements were made on total lung tissue, so direct conclusions about AT2 cells cannot be drawn. More recently, AT2 cells from IPF lungs were characterised by an iron accumulative gene signature, however the absence of classic iron uptake markers (TFR1, TFR2, DMT1) leaves the iron status of AT2 cells ambiguous (119). In COPD research, increased ferroptosis in AT2 cells has been documented in CS-exposed mice, though the specific effects of smoke exposure versus iron-induced pathology remain unclear (209, 210).

To date, studies predominantly focus on lung-iron effects in AMs, with limited research on iron's impact on AT2 cell-specific functions, highlighting a significant gap in the literature. Considering the critical role that AT2 cells play in maintaining alveolar health and their involvement in various pulmonary diseases, coupled with the implications of iron dyshomeostasis in pulmonary pathology, a deeper understanding of their potential overlap is warranted.

1.5 Aims of this Thesis

The central **hypothesis** of this thesis is that iron is essential for AT2 cell health. We believe that disruptions in iron metabolism prevent AT2 cells from carrying out their normal functions, including surfactant homeostasis and alveolar repair. Specifically, given the reliance of AT2 cells on mitochondrial metabolism to generate the substantial energy required for their functions, we hypothesized that MITOIRON deficiency in these cells contributes to their dysfunction, by inadvertently affecting metabolic health. In an attempt to compensate for a lack of iron, AT2 cells may increase iron uptake, maintaining them in a dysfunctional and reparative state, as observed in lung diseases. Furthermore, we propose a loss in AT2 cell MITOIRON may initiate a cascade of inflammatory and fibrotic responses within the alveolar space due to their central role in regulating alveolar health. This research could unveil novel mechanisms and new therapeutic targets that may ameliorate AT2 cell dysfunction in disease. Such targeted therapeutic strategies could have broad applications, potentially transforming treatment paradigms for both IPF and COPD.

The **overall aim** of this research project is to contribute to a fundamental understanding of the role iron plays in AT2 biology in both health and disease.

Research Aims:

1. Develop a tool-kit to study AT2 cell-specific functions *in vitro* and *in vivo* (**Chapter 3**).
2. To explore the role of iron and MITOIRON in AT2 cell specific functions, surfactant homeostasis and alveolar repair, *in vitro* (**Chapter 4**).
3. Expand on how AT2-specific MITOIRON dysregulation alters AT2 cell function *in vivo* and the implications this has on overall lung health (**Chapter 5**).
4. To characterize how iron contributes to the development of AT2 cell dysfunction and senescence following AT2 cell injury *in vitro* and *in vivo* (**Chapter 6**).

2. Materials and Methods

2.1 Materials

2.1.1 Buffers

Table 2.1: Buffer list and their composition

Buffer Type	Buffer Recipe	Use
Stacking Gel Buffer	Tris HCL 1M, @pH 6.8	Western Blotting
Resolving Gel Buffer	Tris HCL @1.5M, @pH 8.8	
5X Sample Loading Buffer	Glycerol, 10% SDS, 10mg bromophenol blue, Tris HCL 1M @6.8, dH ₂ O.	
SDS Running Buffer (10X)	25 mM Tris, 192 mM glycine 0.1 % SDS	
SDS Transfer Buffer (10X)	25 mM Tris, 92 mM glycine 0.1 % SDS	
Tris Buffer saline tween (TBST) (10x)	Tris (24g), 88g NaCL, 10mL Tween-20. Made up to 1L dH ₂ O.	
Stripping Buffer (1X)	Glycine (15g), SDS (1g), Tween-20 (10mL), Made up to 1L dH ₂ O @pH2.2	
Buffer A	Manitol (1mM), Sucrose (350mM), Hepes (50mM), EGTA (5mM), pH7.4	Mitoplast Extraction
Phosphate buffered saline (PBS)(10X)	NaCl (1.45M), NaH ₂ PO ₄ (39mM), NaH ₂ PO ₄ (22.7M)	Protein Extraction
RIPA Lysis Buffer**	Tris HCL (66.6M, @pH8), NaCl (1M), IGEPAL- CA-630 (1%), Sodium deoxycholate (0.5%), SDS (0.1%), up to 200mL	Protein Extraction

** For GFAAS sample preparation, RIPA buffer was prepared in ultrapure water.

2.1.1.1 Murine cell lines

An immortalized murine AT2 cell line isolated from C57BL6, MLE12 cell line, was purchased from ATTC (CRL-2110). RPMI 1640 GlutaMAX media was bought from Gibco biosciences. Foetal bovine serum (FBS), penicillin and streptomycin (P/S) and HEPES were all purchased from Merck. Trypsin ethylenediaminetetraacetic acid (EDTA) purchased from Merck, was used to remove cells from culture flasks and plates. Sterile phosphate-buffered saline (PBS), purchased from Merck was used to wash cells.

Foetal-Liver derived alveolar macrophages (FLAMs) were kindly gifted from Prof. Susan Carpenter, University of California, Santa Cruz. FLAMs are stable long-term and maintain alveolar macrophage-like characteristics, including cellular morphologies, expression profiles

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and functional mechanisms similar to those of primary murine AMs (211). FLAM cells were maintained in RPMI 1640 GlutaMAX media supplemented with 10% FBS, 30ng/ml supGM-CSF and 20ng/ml TGF- β . FLAM cells present as a hybrid adherent/non-adherent population in culture. 1.5 mM EDTA in PBS was utilized to lift adherent FLAMs from culture when passaging and plating.

Human Embryonic Kidney (HEK293T) cells derived from HEK293 cells expressing the simian virus 40 (SV40) large T antigen, were transfected with GM-CSF and generously provided by Prof. Susan Carpenter (University of California, Santa Cruz). These cells stably produce GM-CSF in their supernatant at ~200 ng/ml and can be used as an alternative to commercially available GM-CSF supplements (212). Supernatant GM-CSF (supGM-CSF) was used to maintain FLAM cells in culture.

2.1.1.2 Generation of SLC25A37 and SLC25A28 knockdown via CRISPRi technology

For stable knockdown of *Mfrn1* (*Slc25a37*) and *Mfrn2* (*Slc25a28*) in MLE12 cells, CRISPRi-cas9 technology was utilized. CRISPRi technology is catalytically in-active and functions by targeting and transcriptionally silencing the desired target rather than deletion of the gene. *Mfrn1* and *Mfrn2* were independently targeted using gRNA which were designed to have specific targeting sites and non-target shRNA served as a control (**Table 2.2**). Blasticidin (ant-bl-1; InvivoGen), Puromycin (10054207; Fisher) and Zeocin (R25001; Bio-Sciences) were utilized for positive selection.

Table 2.2: CRISPRi guide sequences

Name	Sequence
Slc25a28_edit_g1	CAGCGCTTCGTATTCCGGGC
Slc25a37_edit_g3	CCTGGTTCCCCACGCCGCCA
F12	F12 Non-targeting CTL 783 gRNA

2.1.1.3 Cell culture materials

Please see below all reagents and materials used in cell culture and their corresponding cat number (**Table 2.3**).

Table 2.3: Cell culture materials and reagents

Material	Cat No:
RPMI Glutamax	61870010
Foetal Bovine Serum (FBS)	F7524-500ML
Penicillin and Streptomycin	P0781-100ML

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Material	Cat No:
HEPES	H0887-100ML
Trypsin EDTA	T4174-100ML
Insulin-transferrin-sodium selenite media supplement	SIG1884-1VL
B-Estradiol	SIGE2758-1G
apo-Transferrin bovine	T1428-100MG
Blasticidin	10648203
Puromycin	10054207
Zeocin	R25001
GM-CSF	130-095-746
TGFβ	PHP143B
DMSO	D2438-50ML
Bleomycin (Sulfate)	CAY13877-10 mg
1R6F Cigarettes	ISO 3308:2012
Ferric Ammonium Citrate (FAC)	F5879
Deferoxamine (DFX)	D9533
Deferiprone (DFO)	Y0001976
NBD-DPPC (1-palmitoyl-2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)-sn-glycero-3-phosphocholine)	810131P
Phorbol 12-myristate 13-acetate (PMA)	16561-29-8

2.1.2 Laboratory Reagents

Please see below all reagents and materials used during general laboratory use and experimental analysis (**Table 2.4**).

Table 2.4: Laboratory reagents and materials

Material	Cat Number	Use
Sodium dodecyl sulphate (SDS)	L3771	Western Blotting
N,N,N',N' Tetramethylethylenediamine (TEMED)	110-19-9	
Ammonium persulphate (APS)	215589-100g	
ProtoGel Acrylamide:Bisacrylamide	EC-890	
WesternBright ECL	K-12045-D50	
Spectra Multicolour Broad Range Protein Ladder	00919330	
Poluvinyldiene difluoride (PVDF)	IPVH00010	
Powdered Milk	Tesco	
BSA	10735094001	
Sodium Azide	S2002-100G	

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Material	Cat Number	Use
Tris	10376743	
β -ACTIN	A2228 (Anti-Mouse) Ab8227 (Anti-Rabbit)	
MFRN1	26469-1-AP	
P21	ab188224	
P53	2524S	
PRO-SFTPC	AB211326	
TFRC	Ab84036	
FASN	C20G5	
EIF2AK1/HRI	MBS2538144	
FTH1	4393S	
Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L)	115-035-003	
Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L)	115-035-003	
Pierce Phosphatase Inhibitor Mini Tablets	A32957	Fluid (with proteins) Storage
Pierce BCA Protein Assay Kit	23225	Protein analysis
Data Physics - disposable needle 2.08mm	SNP-D 208/152	Surface Tension Measurements
RNAse Zap	R2020-250ML	RNA Extraction
PureLink RNA Mini Kit	12183025	
High Capacity cDNA Reverse Transcription Kit, 200 Reactions	4368814	cDNA synthesis
SYBR Green	A25742	RT-qPCR
Alamar blue assay	DAL1025	Cell Viability
Dounce Homogenizer	D8938	Mitochondrial Isolation
Digitonin	CAYM14952-100	
EGTA	045172-100G	
EDTA	17892	
Triton X-100	T8787-50mL	
Nitric Acid	83872.270	Graphite furnace atomic absorption spectrometry
Iron ions standard (1000ppm)	N9303771	
Modifier (0.3% Mg(NO ₃) ₂)	BOI90634	
Iron (Fe) lamp	N3050126	
Oxalic Acid	194131-250g	Heme-Assay
Mouse IL-6 DuoSet ELISA	Catalog #: DY406	ELISAs

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Material	Cat Number	Use
Mouse TNF-alpha DuoSet ELISA	Catalog #: DY410	Mouse Work
Mouse SFTPC / Surfactant Protein C ELISA Kit	MOFI00469	
Mouse IgM (Immunoglobulin M) ELISA Kit	AEFI00751	
4-Hydroxytamoxifen (4-OHT)	H7904	
Injekt-F Fine Dosage Syringe, Volume: 1 mL	10303002	
25G needles 5/8"	300400	
400 mg/kg Tamoxifen citrate mg per kg of diet	TD.130860	
3-0 Silks	W502H	
20G Intravenous Catheters	200-IVS20114	
Anti-CD-45 beads MicroBeads	130-052-301	
CD326 (EpCAM) MicroBeads	130-105-958	
Dispase solution	354235	
Millipore Steriflip	SCNY0020	
Agarose	16520-050 RC-005	
MACS BSA Stock Solution	130-091-376	
Hemolysis buffer	R7757	

2.1.3 Primers Sequences

All primers were designed and purchased from Eurofins Genomics. Please see **table 2.5** for sequence details.

Table 2.5: Primer Sequences

Primer**	Forward Sequence	Reverse Sequence
<i>Actin</i>	TGT TAC CAA CTG GGA CGA CA	GGG GTG TTG AAG GTC TCA AA
<i>Il1β</i>	GGA AGC AGC CCT TCA TCT TT	TGG CAA CTG TTC CTG AAC TC
<i>Il6</i>	ACG ATG ATG CAC TTG CAG	ACT CCA GAA GAC CAG AGG AA
<i>Tnfa</i>	GCC TCT TCT CAT TCC TGC TT	TGG GAA CTT CTC ATC CCT TTG
<i>Slc40a1</i>	CCA TAG TCT CTG TCA GCC TGC T	CTT GCA GCAACT GTG TCA CCG T
<i>Slc25a37</i>	TTG AAT CCA GAT CCC AAA GC	GTT TCC TTG GTG GCT GAA AA
<i>Slc25a28</i>	TCG TCA AGC AGA GGA TGC AGA T	GTT AAA GTG CTC TTG CAG GAA C
<i>Sftpc</i>	CCC TAG TCT TGA GGC TTT CG	GGA ACC AGT ATC ATG CCC TT
<i>Sftpb</i>	TGA GCG CTA CAC AGT TCT CC	CTA TCA GAG GCT CCA GAG CA
<i>Fth1</i>	GCCGAGAACTGATGAAGCTGC	GCAC ACTCCATTGCATTGAGCC
<i>Ftl</i>	CGTCTCCTCGAGTTTCAGAAC	CTCCTGGGTTTTACCCCATTC

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Primer**	Forward Sequence	Reverse Sequence
<i>TfrC</i>	GTTTCTGCCAGCCCCCTTATTAT	GCAAGGAAAGGATATGCAGCA
<i>Cdkn1a (p21)</i>	CTGAGCGGCCTGAAGATT	ATCTGCGCTTGGAGTGATAG
<i>P53</i>	CAAGAGGAATCAGAACCGAGAG	CGTCTTAGGGTGAGGCATTT
<i>Cdkn2a (P16)</i>	CGGGGACATCAAGACATCGT	GCCGGATTAGCTCTGCTCT
<i>Tgfβ</i>	TGATACGCCTGAGTGGCTGTCT	CACAAGAGCAGTGAGCGCTGAA
<i>Pdgfrβ</i>	AAGCCACGCTATGAGATCCG	GCACAACCTCCTTTGCACCTG
<i>Cd36</i>	TCCAGCCAATGCCTTTGC	TGGAGATTACTTTTCAGTGCAGAA
<i>Abca1</i>	AGTTTCGGTATGGCGGGTTT	AGCATGCCAGCCCTTGTTAT
<i>Fasn</i>	GCC ATC CAG ATT GCC CTC AT	CCC AAG GAG TGC CCA ATG AT
<i>Flvcr1a</i>	CCGTCGCCTCGGTATGG	CACTAAAACAGGTGGCAACAAAAA
<i>Flvcr1b</i>	TCGCTTCCTATTGACAGCTATTAACA	CACTAAAACAGGTGGCAACAAAAA
<i>Dmt1</i>	TTGACGCGAGACTTGGAGTGGT	GCTGAGCCAATGACTTCCTGCA
<i>Eif2ak1</i>	TCC CTG TGG GAC TGG ATA A	GTT GCA ACA CTG GCC ATA AC
<i>Hebp1</i>	CAAGTTTGCTACTGTGGAAGTG	CCATGCCGACTCCTTTGT
<i>Marco</i>	GAAGACTTCTTGGGCAGCAC	CTTCTTGGGCACTGGATCAT
<i>Pprγ</i>	GTA CTG TCG GTT TCA GAA GTG CC	ATC TCC GCC AAC AGC TTC TCC T
<i>Cd11c</i>	TGC CAG GAT GAC CTT AGT GTC G	CAG AGT GAC TGT GGT TCC GTA G
<i>Fgfr2</i>	GTCTCCGAGTATGAGTTGCCAG	CCACTGCTTCAGCCATGACTAC
<i>Aqp5</i>	TCTACTTCACCGGCTGTTCC	TCCGCTCCTCTCTATGATCTTC
<i>Pdpr</i>	GAGGTGCCTTGCCAGTAGAT	CATCCCTGACTGGCCTTTCTT
<i>Krt8</i>	TGGAGCAGCAGAACAAGATG	CCGCCTAAGGTTGTTGATGT

** Species = *Mus musculus* for all primer sequences

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Culture of MLE12

MLE12 were cultured and maintained in RPMI 1640 medium supplemented with 2% (v/v) FBS, 1% (v/v) P/S, L-glutamine (2mM), Hepes (10mM), Insulin-transferrin-Sodium Selenite (2.5mL), transferrin, β -estradiol (100 μ M) and hydrocortisone (100 μ M), which will now be referred to as “HITES medium”. Cells were seeded when 80-90% confluency was obtained. In a laminar flow hood, complete medium was removed from the flask and discarded. Cells were subsequently washed with PBS and removed from the flask utilizing Trypsin-EDTA. Complete medium was added to neutralize the trypsin. Cells were then pelleted in a centrifuge at 1500xg for 5minutes.

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Finally, cells were seeded at 0.2×10^6 cells/ml in 30mL of complete medium in a T175 flask and stored in an incubator containing 5% CO₂ at 37°C. For experiments, cell density varied depending on the experiment and the functional readout under investigation, please see **table 3.1** for cell densities used in culture.

2.2.1.2 Culture of HEK293T expressing recombinant GMCSF

HEK293T cells were cultured in DMEM (Dulbecco's Modified Eagle Glutamax Medium) with 10% FBS and grown to 80-90% confluency in T175 flasks. Upon reaching confluency, cells were passaged using Trypsin-EDTA, neutralized with 10% FBS in DMEM after 10 minutes and collected by centrifugation at 1500xg for 5 minutes. The cell pellet was resuspended in fresh media and replated at the required density (**Table 3.1**). After 3 days, the supernatant was collected, centrifuged, pooled, filter-sterilized and aliquoted for use in FLAM medium supplementation.

2.2.1.3 Culture of foetal derived alveolar macrophages

FLAM cells were cultured and maintained in RPMI Glutamax 1640 medium supplemented with 10% (v/v) FBS, 30ng/ml supGM-CSF and 20ng/ml TGF- β . Media was changed every two-three days to replenish nutrients and levels of GM-CSF and TGF- β . Once cells reached 80-90% confluency they were passaged by splitting the cells in a 1:2 split. Suspension cells were removed and placed in a 50mL falcon, while adherent cells were lifted utilizing 1.5 mM EDTA in PBS for 10 min at 37°C. Once lifted, both cell suspensions were combined and pelleted in a centrifuge at 1500xg for 5 minutes. Supernatant was discarded and FLAMs resuspended in 5mL of fresh media. Cells were subsequently counted and cellular density determined. FLAMs were seeded at the required density for the experimental readout (**Table 3.1**).

2.2.1.4 Generation of stable knockdown of SLC25A37 and SLC25A28 in MLE12 cells utilizing CRISPRi technology.

Of note CRISPRi cells were generated by a previous laboratory member from the Cloonan laboratory. Briefly MLE12 cells were transfected with Cas9/dCas9 constructs, which were kindly provided by the Carpenter Lab (UC, Santa Cruz). Cells were then cultured with 20 μ g/ml of blasticidin following transfection to allow for selection of the stable transfected cell population. Cells were then transfected with GFP machinery allowing for cells carrying a functional CRISPRi Cas9 system to be visualized via fluorescent microscopy. GFP fluorescence was then analysed to confirm the transfections was successful. Cells were then transfected with mitoferrin 1 or mitoferrin 2 guide RNAs (gRNA) to induce a stable knockdown of the selected genes; for guide sequences see **Table 2.2**. Knockdown efficiency of the guides

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was assessed via RT-qPCR. Cells were then cryopreserved, as described below. When thawed, cells were cultured and maintained in HITES medium supplemented with blasticidin (20µg/mL), zeocin (500µg/mL) and puromycin (1µg/mL) for 10-14 days before use. Similarly, CRISPRi cells were split 1in4 every 3-4 days once they reached 80-90% confluency into fresh culture flasks and returned to sterile incubators.

2.2.1.5 Cryopreservation

All cells were cultured in sterile culture flasks until 80% confluent. Cells were pelleted, as described above and then resuspended in complete RPMI media to be counted, utilizing a haemocytometer. Between 1 and 2×10^6 cells/ml were frozen in their corresponding cell culture media containing 10% DMSO and 30% FBS (**Table 2.6**). DMSO was added dropwise to the resuspended cells and aliquoted into 1.5mL cryovials. The cryovials were cooled slowly at an optimal freezing rate of $-1^\circ\text{C}/\text{minute}$. Once frozen, cells were stored in the -80°C freezer for long term storage.

Table 2.6: Cell culture Densities

Cell Type	Density	Conditions	Duration of Culture
MLE12 and CRISPRi Cells	@ 0.4×10^6 cells/ml	6-well (Protein) 12-well (RNA)	Max 48hrs
	@ 0.4×10^5 cells/well	96-well (Scratch, NBD-DPPC uptake, alamar blue Assays)	
FLAM Cells	@ 0.5×10^6 cells/ml	6-well (Protein) 12-well (RNA)	Max 48hrs
	@ 0.4×10^5 cells/well	96-well (NBD-DPPC uptake, alamar blue assays)	
HEK293T	@ 9×10^6 cells/50mL	T175	72hrs
All cells	@ $1-2 \times 10^6$ cells/ml	30% FBS, 10% DMSO and 60% complete medium	n/a

2.2.1.6 Generation of Cigarette Smoke Extract (CSE)

Standard 16RF research cigarettes (The University of Kentucky), devoid of filters, were smoked using a Masterflex L/S Easy-Load 3 pump set at 1500xg. The smoke was then infused into RPMI media at a ratio of one cigarette per 10 mL of media using a peristaltic pump. The resulting CSE was filter sterilized designated as a 100% CSE solution and stored at -80°C .

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Each CSE batch underwent testing on the MLE-12 cell line before use, employing a standardized dosage rate to ensure consistency across batches.

2.2.1.7 Cell Treatments

Please see **Table 2.7** for details on all treatments used during *in vitro* experiments. Varying incubation times were utilized during experimentation as outlined in the results figure legends.

Table 2.7: Cell culture treatments

Treatment	Reconstituted	Working Concentration	Treatment (hrs)
Bleomycin (sulfate) salt	PBS	5µg/mL – 10µg/mL	24-48hrs
Ferric Ammonium Citrate (FAC)	Sterile Water	100µM	24hrs
Deferiprone (DFP)	Sterile Water	10µM	24hrs
Deferoxamine (DFX)	Sterile Water	10 µM	24hrs
Cigarette Smoke Extract	RPMI	5-10%	24-48hrs
Phorbol 12-myristate 13-acetate (PMA)	DMSO	10nM	2-4hrs

2.2.3 Assessment of Protein Expression by Western Blotting

2.2.3.1 Cell Lysis and Harvesting

Firstly, medium was removed from the cell culture dish. Adherent cells were then washed with 1mL of ice cold 1X PBS to remove excess media. An additional 1mL of PBS was added and cells were scrapped using a cell scraper. The PBS containing the cells was then transferred to a 1.5mL eppendorf tube and centrifuged for 5minutes @300xg. Cells were subsequently washed and re-pelleted before being re-suspended in 50µL of RIPA lysis buffer containing protease inhibitors and placed on ice for 15mins. Cells were centrifuged at 18,400xg for 10mins. The supernatant was then isolated and transferred into newly labelled epis and stored at -20°C until a protein quantification could be performed.

2.2.3.2 Bicinchoninic acid (BCA) assay:

A BCA protein assay was carried out as described by the manufacturer's instructions. Briefly, samples were defrosted on ice and while defrosting, the standard solutions were then prepared and 25µL of each standard sample was pipetted onto a 96-well plate in duplicate. The plate was then incubated on ice while the BCA reagent mix was prepared. Reagent mix

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was prepared in the recommended ratio of 50 (A): 1 (B). Once prepared, 200 μ L of the BCA reagent mix was added to each individual well. The plate was then covered in tinfoil to protect the reagent from light exposure and incubated @37°C for 30minutes. After the incubation period the plate was allow to cool to room temperature and then the absorbance was read on a plate reader @562nm.

2.2.3.3 Western blot preparation and conditions

The desired protein concentration for the samples (50 μ g protein/sample for all samples unless detailed otherwise) were mixed with 1X sample buffer (see **Table 2.1**); containing 50mM DTT and heated @95°C for 5minutes. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to visualize and separate the proteins. Samples were run on a 8-15% gradient, see **Table 2.8** for gel recipes. Each gel was run with a molecular weight marker as a reference. Each gel was run through the stacking for approximately 30minutes between 60-80V gradient. Samples were then run through the resolving gel for a further 60minutes between 100-120V gradient.

Table 2.8: Stacking and running gel compositions

Reagent	Resolving Gel (10mL)				Stacking Gel (4mL)
	8%	10%	12%	15%	
dH ₂ O	4.6mL	4mL	3.3mL	2.3mL	2.7mL
30% Acrylamide	2.7mL	3.3mL	4mL	5mL	670 μ L
Tris HCL 1.5M pH 8.8	2.5mL	2.5mL	2.5mL	2.5mL	-
Tris HCL 1M pH 6.8	-	-	-	-	500 μ L
10% SDS	100 μ L	100 μ L	100 μ L	100 μ L	40 μ L
10% APS	100 μ L	100 μ L	100 μ L	100 μ L	40 μ L
TEMED	6 μ L	4 μ L	4 μ L	4 μ L	4 μ L

The proteins were then transferred via the wet transfer to PVDF method. A small basin was filled with SDS-Page transfer buffer (see **Table 2.1**). Within the basin a black sponge was placed on the black side of the sandwich cassette and two pieces of filter paper stacked directly on top. The gel was then placed on top of the filter papers. PVDF membrane was cut to a rectangular shape measuring to the same size as the gel containing the resolved proteins. The membrane was then activated in 100% methanol and placed on top of the gel. An additional two pieces of filter paper were placed on the membrane, followed by a black sponge.

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Utilizing a roller, air-bubbles were removed from the stack and the cassette was closed and placed into the tank containing transfer buffer and an ice pack, to avoid over-heating during this step. The transfer run was set @200mA for two hours.

Once transferred blots were then blocked in either 5% (w/v) milk powder for 1hr at room temperature. The membrane was then incubated in the desired primary antibody at the desired antibody concentration overnight at 4°C, see **table 2.9** for antibody concentration details. The next day the membrane was then washed in TBST three times, at 15 minutes per wash. Once washed, the membrane was incubated in the suitable secondary antibody for one hour at room temperature. After the hour the membrane was washed a further two times, at 15 minutes per wash in TBST. For visualisation, chemiluminescent substrate was diluted in the recommended ratio of 50 (A): 1 (B). The membrane was then incubated in the substrate for approximately 30seconds before visualisation on the Chemi-Doc MP gel imaging system. Blots were then analysed with the Image Lab software, BioRAD.

Table 2.9: Antibody dilutions

Primary Antibody	Blocking	Secondary Antibody
β-actin (1:10000 in 5% Milk)	5% Milk	Anti-Rabbit (1:10000 5% Milk)
MFRN1 (1:600 in TBST)	1% Milk	Anti-Rabbit (1:2500 TBST)
P21 (1:1000 in 5% Milk)	5% Milk	Anti-Rabbit (1:10000 5% Milk)
P53 (1:1000 in 5% Milk)	5% Milk	Anti-Mouse (1:10000 5% Milk)
Pro-SFTPC (1:1000 in 5% Milk)	5% Milk	Anti-Rabbit (1:10000 5% Milk)
TFRC (1in1000 in 5% Milk)	5% Milk	Anti-Rabbit (1:2500 in 5% milk)
FASN (1in1000 in 5% Milk)	5% Milk	Anti-Rabbit (1:2500 in 5% milk)
EIR2AK1/HRI (1in600 in 5% Milk)	5% Milk	Anti-Rabbit (1:2500 in 5% milk)
FTH (1in1000 in 5% Milk)	5% Milk	Anti-Rabbit (1:2500 in 5% milk)

2.2.3.4 Western blot stripping

For stripping the PVDF membrane was washed in 10mL of stripping buffer for 5-10mins twice. The membrane was then washed in PBS for 10mins and then subsequently in 1X TBST for an additional 10mins. The membrane was then blocked in either 5 % milk for 1 hour depending on the protein being detected. The membrane was then washed in TBST for 15mins. Once washed, the membrane was then incubated in the desired primary antibody over night at 4°C.

2.2.4 Detection of Secreted Proteins by Enzyme-Linked Immunosorbent Assay (ELISA)

DuoSet® ELISA kits for the pro-inflammatory cytokines IL-6 and TNF- α were procured from RandD Systems. Additional ELISAs utilized include mouse SftpC (mSFTPC) and mouse Immunoglobulin M (mIGM) were purchased from AssayGenie. The assays were performed following the manufacturer's detailed protocol. Briefly, a standard curve was generated using serial dilutions of known concentrations of IL-6, TNF- α , mSFTPC and IGM standards provided in the kit. Sample concentrations were then accurately extrapolated from this standard curve. A four-parameter logistic (4PL) curve fit was applied to the standard curve data, ensuring precise quantification of the cytokine or protein levels within the samples.

2.2.5 Analysis of mRNA Expression in Cultured Cells by Quantitative Real Time Polymerase Chain Reaction (RTqPCR)

2.2.5.1 RNA Extraction

Briefly, media was removed from the culture dish and discarded. Cells were then washed with PBS and 350 μ L of lysis buffer was pipetted directly into the well. Previously, 70% molecular grade ethanol was prepared in RNase free water and an equal amount of 70% ethanol was added to the well. Next, the lysate was collected and transferred into a RNA mini spin column. Samples were then centrifuged @12,000 g for 15seconds. The filtrate was then discarded and 700 μ L of wash buffer-I was pipetted into the spin tube. Samples were then centrifuged again @12,000 g for 15seconds. The elute was discarded and 500 μ L of wash buffer-II was pipetted into the spin column. Samples were centrifuged again for @12,000 g for 15seconds. An additional 500 μ L of wash buffer-II was added and the previous step repeated. Columns were then placed in a fresh collection tube and spun @12,000 g for 2minutes, to dry the column. Once dry, columns were placed in RNase free Eppendorf tube. 30 μ L of RNase free water was directly pipetted onto the column and allowed incubate on the column for 1minute at room temperature. All tubes were then centrifuged @12,000 g for 2minutes. Finally, the yield (ng/ μ L) and quality (A260/280) of the eluted RNA was quantified utilizing a Nanodrop Spectrophotometer.

2.2.5.2 Complementary DNA synthesis

RNA was transcribed into complementary DNA (cDNA) utilizing the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. Briefly, Master Mix was

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prepared following the below composition (**Table 2.10**). 10µL of sample containing between 50ng/µL - 100ng/µL and 10µL of Master Mix was added to each tube. The samples were then placed in a thermocycler and run at the following conditions: Annealing 10min @25°C, Elongation 120mins @37°C, Enzyme inactivation 5minutes 85°C. Once synthesised, DNA was diluted 1in5 in dH₂O and stored @4°C .

Table 2.10: cDNA 2X master mix composition (per sample)

Component	Reagent (RT+)	Reagent (RT-)
10X RT Buffer	2.0 µL	2.0 µL
25X dNTP Mix (100 mM)	0.8 µL	0.8 µL
10X RT Random Primers	2.0 µL	2.0 µL
MultiScribe™ Reverse Transcriptase	(0.5µL)	0 µL
Nuclease-free H ₂ O	4.7 µL	5.2 µL
Total	10.0 µL	10.0 µL

2.2.5.3 qPCR using SYBR® Green

RT-qPCR was performed using SYBR® Green qPCR MasterMix alongside with mouse gene-specific forward and reverse primers (**Table 2.5**). Primer mixes were prepared for each target containing both the reverse and forward primer at a final concentration of 10µM for both. Reaction mix was prepared for each target gene containing SYBR (5µL), Primer Mix (0.3µL) and dH₂O (0.7µL). For each well. 6µL of reaction mix was added and an additional 4µL of cDNA. Of note, all samples and controls were run in technical triplicates. The 96-well plate was then sealed with adhesive film and centrifuged @500xg for 1min. The qPCR run was then performed on Applied Biosystems 7900 HT machine, see **Table 2.11** for conditions. To ensure specificity and stability of the primers a melt curve was run in conjunction with the PCR run. Relative expression for each sample was determined via the Comparative C_T method (213). The C_T value of the target gene was normalized to the C_T value of a house keeping gene. Once normalized, the relative expression compared to the control was investigated and the fold-change of gene expression calculated.

Table 2.11: Fast cycling mode (primer T_m≥60°C)

Step	Temperature	Duration	Cycle
UDG Activation	50°C	2 minutes	Hold
Dual-lock™ DNA polymerase	95°C	2 minutes	Hold
Denature	95°C	1 seconds	40
Anneal/extend	60°C	30 seconds	

2.2.6 Quantification of Iron levels

Iron levels in as little as 5000 cells can be measured by graphite furnace atomic absorption spectroscopy (GFAAS) using a PinAAcle 900Z Atomic Absorption Spectrometer machine purchased from PerkinElmer.

2.2.6.1 Mitochondrial isolation

Utilizing a 80% confluent 10cm dish, media was removed and cells were washed with buffer A (**Table 2.1**). Cells were subsequently scraped into fresh 5mL of cold extraction buffer A and centrifuged @700xg for 5 minutes at 4°C. The pellet was then washed with another 5ml of buffer A and re-pelleted. The supernatant was then removed and the cells were resuspended in 1mL of buffer A (**Table 2.1**) containing 0.5mM EDTA, to ensure the removal of excess iron in the buffers. Samples were then incubated on ice for 20mins to induce cell swelling. The resuspended pellet was then transferred to a 1mL dounce homogenizer which was chilled on ice. For epithelial cells, samples were homogenized between 80-100 times. The homogenized solution was then transferred to a 1.5mL eppendorf tube and centrifuged @700xg for 10 minutes at 4°C. The supernatant was transferred to a pre-cooled 1.5mL epi and centrifuged for 11,000xg for 20mins at 4°C. The cytosolic fraction was then carefully pipetted off into a new epi and stored @-20°C until the samples were analysed. The pellet, which represents the crude mitochondrial fraction, was either resuspended in 50µL of RIPA buffer containing protease and phosphatase inhibitors, which can be used for GF-AAS measurements or utilized to generate mitoplasts (see below).

2.2.6.2 Mitoplast isolation

Mitoplasts were generated from crude mitochondrial fraction (214). Mitoplasts consist of the inner mitochondrial membrane or matrix enclosed mitochondria with no outer membrane. The crude mitochondrial fraction was washed and pelleted @11,000xg for 5mins @4°C four times. The supernatant was carefully pipetted off, ensuring the pellet was dry. The pellet was then resuspended in buffer A containing 0.5% w/w digitonin and incubated on ice for 15mins. The samples were then centrifuged @11,000xg for 10mins @4°C. The pellet was then washed twice in buffer A. The pellet was then utilized for the measurement of mitochondrial iron levels via GF-AAS.

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2.2.6.3 Sample digestion

Firstly samples were digested and prepped before analysis on the machine. Media was removed from the culture dish and kept for iron measurements when needed. Cells were then scraped into molecular grade PBS and pipetted into an iron free Eppendorf tube. Cells were then pelleted @13,500xg for 5minutes at room temperature. The supernatant was subsequently removed and the step was repeated to wash cells. Once pelleted for the second time, a fraction of the sample was removed for protein quantification via BCA analysis. The sample was then resuspended in 100µL of RIPA buffer (**Table 2.1**). For cell culture experiments samples were prepared in technical triplicates. An equal amount of 50% nitric acid was added to digest the sample. The sample was then heated @70°C for 2hrs in the fume hood. Tubes were then allowed to cool to room temperature before they were centrifuged @6000xg for 5minutes at room temperature. Samples were kept in the -80°C until further analysis.

2.2.6.4 GF-AAS set up

To set-up the GF-AAS the computer, 99.9% pure argon gas and machine are all turned on in that sequence. The Synergistix for AA software is opened and the lamp is turned on and allowed to heat up. The energy levels must reach about 72 before continuing. While the lamp heats up, samples and standards are prepared. All samples were diluted in 0.2% Nitric acid. Standards are prepared at 10ppb and 50ppb from the Iron ion standard (1000ppm). The modifier (0.3% Mg(NO₃)₂) and prepared standards are all placed in specific positions in the sampling holder.

2.2.6.5 GF-AAS use

With GF-AAS, the auto-sampler directly injects the sample into a graphite tube. The sample is then vaporized in a series of steps which have been previously investigated and programmed into the machine. The sample is then atomized within the tube. The free atoms then absorb the iron (Fe) light path @248.33nm, which allows for a direct readout of the atomic concentrations of the element of interest Fe. The method used for the atomisation of iron in GF-AAS includes four steps with one cleaning step (see **Table 2.12** for details).

Briefly, during the first step, the drying step, samples are evaporated leaving a solid residue in the tube. The time of this step is determined by the amount of sample (µL) being pipetted into the tube, with a larger sampling amount needing a longer dry time. The selected temperature must ensure that the sample is not heated too quickly so as to avoid splatter and waste of the sample. After drying, ashing occurs. This is a pyrolytic method in which the sample is heated

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to a high enough temperature to allow for the removal of inorganic and organic materials from the sample. This temperature must not be too high to avoid the loss of iron before atomisation. Finally, the molecules are turned into atoms and are able to absorb the light from the Fe cathode lamp.

Before running the samples a calibration curve is run of the standards and their known Fe content; generating a standard curve with a corresponding correlation co-efficient, of note 0.098 was the cut-off point for the correlation co-efficient to ensure accurate reading. Each sample is then run in triplicate as the machine calculates the mean and extrapolates the corresponding Fe $\mu\text{g/L}$ for each sample. Each sample is run in technical triplicate on the machine and an average of the measurements is taken and normalised to the protein (mg) present in the sample. To insure the machine is operating efficiently throughout the run a quality control step is added after every 10th sample of a known standard, ensuring the readout is still robust and sensitive.

Table 2.12: Heating steps for atomisation of samples

Step	Temperature (°C)	Time (s)	Internal Flow (ml/min)
Drying 1	110	35	250
Drying 2	130	30	250
Ashing (Pyrolysis)	1400	20	250
Atomisation	2100	3	0
Cleaning	2450	3	250

2.2.7 Measurement of Heme Levels

Intracellular and BALF heme concentrations were measured using a fluorescence-based method, as described previously (215). For this cellular measurement, cells were plated @0.5x10⁶cells/mL in a 10cm culture dish. Briefly, media was removed from the culture dish and cells were washed and scraped into PBS. Cells were then pelleted by centrifugation @5000xg for 5minutes @4°C. The samples were then washed and re-pelleted and transferred to an iron-free 1.5mL Eppendorf tube. A fraction of the sample was removed for protein determination via BCA analysis, with the remaining sample to be re-pelleted as previously. The pellet was then divided into two equal aliquots. For both aliquots, 500 μL 2M oxalic acid was added. For BALF, samples were centrifuged @1000xg for 5minutes. Samples were divided into two 100 μL aliquots and 200 μL of 2M oxalic acid added to both. Next, one aliquot was left at room temperature, for background measurements. The other aliquot was then heated for 30minutes @95°C. This heating step allows the release of iron from hem and the

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subsequent generation of PPIX. The background measurement measures naturally endogenous and free porphyrins (215).

Once heated, samples were then centrifuged for 10 minutes @1000xg at 4°C to remove debris. 200 µL of the supernatant was then pipetted in duplicate into a black 96-well plate and the fluorescence was assessed at ex 400nm/emission 620nm/excitation. For analyses, the fluorescence for each boiled aliquot was subtracted from the corresponding un-boiled aliquot. To estimate intracellular heme concentrations the following equation was followed: 1 µM heme = 15,200 fluorescence units at 622nm (215). Samples were then normalized to their corresponding protein concentrations.

2.2.8 Surface Tension Measurements

The surface and interfacial activity of MLE12 supernatant (supMLE12) and mouse pulmonary surfactant (PS) were evaluated using the pendant drop shape analysis method, as previously described (209, 216). In brief, droplets of supMLE12 (~35 µL) and mouse PS (~30 µL) in PBS are dispensed into a droplet from a 2.08 mm nozzle. The density of the liquid (kg/m³) is calculated and inputted into the optical contact angle measuring and contour analysis system of the OCA software. The surface tension (IFT mN/m) is measured in zero field (γ_0) and calculated based on the droplet's shape (209, 216, 217). The surface tension (IFT mN/m) is determined based on the shape of the droplet, with a change in droplet shape equating to a change in surface tension. Each measurement was conducted with a minimum of four droplets to ensure accuracy.

2.2.9 Cell Culture Assays

2.2.9.1 Alamar blue assay

An Alamar Blue assay was conducted to assess cellular viability and proliferation, following the manufacturer's protocol. This colorimetric and fluorometric method relies on the reduction of resazurin, a non-fluorescent blue dye, to resorufin a fluorescent pink compound, by metabolically active cells. After adding the Alamar Blue reagent to cell cultures and incubating for 3-4hrs, the resulting colour change or fluorescence was measured at 560em, 590exm. The media used in the corresponding experiment served as a blank measurement, which was subtracted from each value. Each measurement was performed in technical triplicate.

2.2.9.2 "Surfactant-Uptake" Assay

Surfactant composition primarily consists of approximately 80% phosphatidylcholine (PC), with Dipalmitoyl-phosphatidylcholine (DPPC) constituting 50-60% of that PC fraction. To indirectly assess surfactant uptake, consumption of Nitrobenzoxadiazole-labelled DPPC (NBD-DPPC), a fluorescent variant of DPPC (16:0-12:0 NBD PC, Cat. no. 810131P, Avanti, Sigma-Aldrich) was measured. Cells were seeded in a 96-well black-well clear-bottom plate at the desired density. Following cell adhesion and relevant treatments, cells were exposed to 30 ng/ml of NBD-DPPC and then incubated in a dark environment with 5% CO₂ at 37°C for 2 hours. Post-incubation, the plate underwent two gentle PBS washes, followed by resuspension in PBS. Fluorescence readings were taken from the bottom of the plate using a plate reader at 464 nm (excitation) / 531 nm (emission).

Analysis involved subtracting the mean background fluorescence (media and NBD-DPPC without cells) from the signal of cells treated with NBD-DPPC. Raw fluorescence values were normalized based on the protein content obtained from BCA analysis.

NBD-DPPC uptake was visualized via confocal microscopy. Cells were seeded in glass-bottom dishes and incubated overnight for adhesion. Cells were then treated with 30 ng/ml NBD-DPPC and incubated at 37°C with 5% CO₂ for 1 hour in the dark. After washing with PBS, cells were supplemented with phenol red-free HITE medium containing 3 mg/ml Hoechst and incubated for 15 minutes. The medium was replaced with fresh phenol free HITES medium before imaging on a Leica SP8 confocal microscope.

2.2.9.3 “Surfactant-Secretion” assays

To assess surfactant secretion in MLE12 cells, the cells were plated and allowed to adhere overnight. The next day, cells were washed with PBS, and 1 mL of fresh media was added to each well. Cells were then stimulated with PMA for 2-4 hours. Post-stimulation, the media was collected and centrifuged at 1500xg for 5 minutes. The supernatant was aliquoted with or without protease and phosphatase inhibitors for downstream analysis. For surface tension measurements, inhibitors were not used to avoid interactions with lipids, as maintaining the integrity of surfactants is crucial for accurate readings. Surface tension measurements were conducted as previously described above. For the phosphatidylcholine (PC) assay, fold-change was determined by comparing the initial supernatant (0 hours pre-stimulation) to the subsequent increase in PC levels using a coupled enzyme assay (Sigma MAK049). Specifically, phosphatidylcholine was quantified by enzymatically converting it to choline, which is oxidized to produce hydrogen peroxide. This reacts with a fluorogenic probe, generating a fluorescent signal proportional to phosphatidylcholine levels, measured at 535ex/587em.

2.2.9.4 Wound healing assay

A wound healing assay was conducted using the Incucyte® and Woundmaker tool. MLE12 cells were plated at the required density, allowed 24-48 hours to form a monolayer and then scratched with the Woundmaker. Images were taken every 8 hours for 48 hours. The Incucyte® Scratch Wound Analysis Software measured wound width, confluence, and relative wound density over time. A wound mask was initially generated to identify the wound edge, and an algorithm calculated wound confluence (%), indicating the fractional area occupied by cells. For some experiments where the software was unavailable, confluency was measured directly and compared to previous wound closure data, showing a $\leq 10\%$ variation, confirming its adequacy for representing wound closure. Each condition was performed in technical triplicate, with average readings taken at each time point.

2.3 In Vivo Methods

2.3.1 Mice

2.3.1.1 Lox Cre system

Mfrn1^{loxP/loxP}, *Mfrn2*^{loxP/loxP} and *Mfrn1/2*^{loxP/loxP} mice were kindly provided Prof. Diane M. Ward, University of Utah. *Sftpc*^{CreERT2+/-} mice were shared from Dr. Brigid Hogan. To construct mice with a tamoxifen-inducible knockout system of *Mfrn1* and *Mfrn2* genes specifically in AT2 cells, *Mfrn1/2*^{loxP/loxP} mice were crossed to *Sftpc*^{CreERT2+/-} mice generating *Mfrn1*^{loxP/wt} *Mfrn2*^{loxP/loxP} *Sftpc*^{CreERT2+/-} mice (hereafter termed *Mfrn1*^{+/-} *Mfrn2*^{iAT2} mice or Heterozygous HetKO mice) (Figure 2.1).

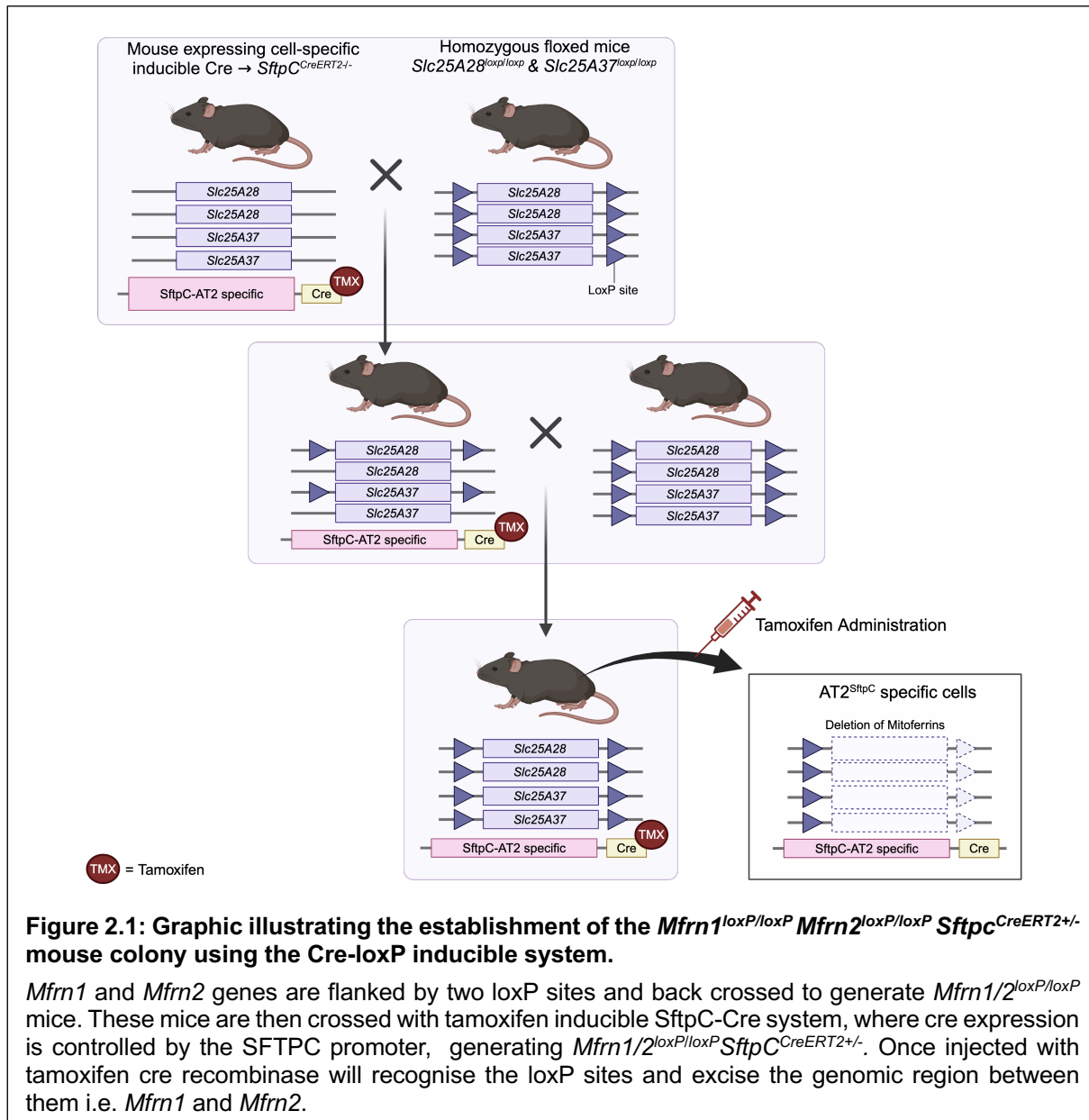


Figure 2.1: Graphic illustrating the establishment of the *Mfrn1*^{loxP/loxP} *Mfrn2*^{loxP/loxP} *Sftpc*^{CreERT2+/-} mouse colony using the Cre-loxP inducible system.

Mfrn1 and *Mfrn2* genes are flanked by two loxP sites and back crossed to generate *Mfrn1/2*^{loxP/loxP} mice. These mice are then crossed with tamoxifen inducible *Sftpc*-Cre system, where cre expression is controlled by the SFTPC promoter, generating *Mfrn1/2*^{loxP/loxP} *Sftpc*^{CreERT2+/-}. Once injected with tamoxifen cre recombinase will recognise the loxP sites and excise the genomic region between them i.e. *Mfrn1* and *Mfrn2*.

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Mfrn1 (*flox*) and *Sftpc* (*Cre*) are both located on chromosome 8, resulting in reduced meiotic recombination and lowering the probability of achieving a homozygous *Mfrn1*^{-/-} genotype to approximately 1 in 500. Despite this challenge, a former member of the Cloonan Lab, Ziling Huang, successfully established the *Mfrn1*^{loxP/loxP} *Mfrn2*^{loxP/loxP} *Sftpc*^{CreERT2+/-} mouse colony (referred to as Real - RDKO mice) at Cornell University, New York. Embryos from this colony were frozen and shipped to Ireland, where they were rederived in the Comparative Medical Unit (CMU) in 2022/2023.

Previously, at the fifth to sixth week of age mark, six intraperitoneal tamoxifen (TMX) injections dissolved in sunflower seed oil were administered, as described previously (81), to allow for recombination by CreERT2. In alignment with the Health Products Regulatory Authority (HPRA) 3Rs principles (Reduce, Refine, Replace), tamoxifen (TMX) administration at Trinity was optimized for the establishment of the RDKO line, as detailed in **Chapter 5**. The refined protocol involves five subcutaneous TMX injections (100 mg/kg) dissolved in Chen oil (4:1 Sunflower Seed Oil: Castor Oil) over two weeks, followed by four weeks of TMX diet supplementation. The number of injections and duration of dietary supplementation were adjusted as necessary; further details can be found in **Chapter 5**. *Mfrn1/2*^{loxP/loxP} and *Sftpc*^{CreERT2+/-} mice were used as control for experiments.

Similarly *Fasn*^{iΔAEC2} were generated utilizing the Cre-loxP inducible system as described previously (209). Briefly, *Fasn*^{loxP/loxP} homozygous mice were crossed with *Sftpc*^{CreERT2-/-} mice generating *Fasn*^{loxP/loxP} *Sftpc*^{CreERT2+/-} mice (hereafter termed *Fasn*^{iΔAEC2} mice). *Fasn*^{+/+} *Sftpc*^{CreERT2+/-} littermates served as controls.

All animal experiments and procedures described in this thesis were approved by the Institutional Animal Care and Use Committee at Weill Cornell Medicine or the Health Products Regulatory Authority (HPRA) under project authorization AE19136/P175 and were conducted in compliance with all relevant ethical regulations.

2.3.1.2 Genotyping of *Mfrn1* and *Mfrn2* deletion in murine AT2 cells

For genotyping, ear-clipping from breeding colonies were obtained after weaning and sent to Transnetyx for genotyping. For confirmation of stable knockdown in experiments carried out in Trinity college Dublin, RNA was extracted from primary AT2 cells isolated from RDKO and Control (*Mfrn1/2*^{fl/fl} control) mice and knockdown efficiency tested by RTqPCR.

2.3.2 Treatments

2.3.2.1 Bleomycin treatment

Bleomycin instillations were given at 12 weeks to sex and weight matched mice. Isoflurane was delivered as a form of inhalant anaesthetic. Once mice were anaesthetised, Bleomycin (Dose: 1 µg/µl/g body weight in 50 µl PBS; 13,877, Cayman Chemical Company, MI, USA) or PBS (50µl) as control was delivered via intratracheal instillation with the assistance of an otoscope, as described previously (81). From the fourth day after bleomycin insult, the weight of the mice was recorded every second day. Mice were then euthanized at different time points for sample harvest. Details of when samples were harvested can be found in figures and their corresponding figure legends.

2.3.2.2 Smoke exposure

Data-sets and samples from smoke exposed mice were utilized in this project, data and sample collection was performed by Li-Chao Fan in Cornell, New York. Sample processing and data analysis, was carried out by Sarah Kenny (me). A classic smoke model was employed in this study, *Fasn*^{iΔAEC2} mice or their control *Fasn*^{-/-} *Sftpc*^{CreERT2+/-} littermate mice, aged at 8-12 weeks were exposed to cigarette smoke (CS), as described previously (209). Briefly, using a TE-10 inhalation exposure apparatus (Teague Enterprises). The exposure involved 100 3R4F cigarettes (obtained from the University of Kentucky Center for Tobacco Reference Products) with an average total particulate matter concentration of 150 mg/m³. Mice were exposed for 2 hours daily, 5 days per week, over a period of 1.5 to 8 months (209).

2.3.2.3 Tamoxifen injection

Tamoxifen injections were given at 5-6 weeks to sex and weight matched mice. 4-Hydroxytamoxifen (4-OHT) was prepared by dissolving 4-OHT powder in 100% ethanol at a concentration of 40 mg/mL under a fume hood. The solution was aliquoted and stored at -20°C until needed. On the day of injection, aliquots were thawed and vortexed for 10–15 minutes until fully dissolved. Chen oil was prepared as a 4:1 mixture of sunflower seed oil and castor oil, stored at room temperature. The required dose (100 mg/kg) was calculated based on the total weight of the mice. The appropriate amount of 4-OHT solution in ethanol was mixed with Chen oil to achieve a final concentration of 20 mg/mL. A vehicle control solution was prepared using Chen oil and ethanol without 4-OHT. Solutions were vortexed (2–3

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minutes) until homogeneous. Ethanol was removed by vacuum centrifugation. Each mouse was injected slowly, with the needle held in place briefly, post-injection to prevent leakage.

2.3.3 Downstream *in vivo* Analysis

2.3.3.1 Isolation of murine AT2 cells via MACs® separation

AT2 cells were isolated from mouse lung utilizing MACs® separation, as described previously (81). Briefly, mice were euthanized and the lungs were perfused in PBS. Once perfused, lungs were then digested in 2mL dispase at room temperature for 45minutes and then inflated with 1.5mL dispase (Cat:354235, BD Biosciences), which was fixed by 0.5 ml 1% low-melting point agarose in trachea (Cat:16520–050, Invitrogen). Lungs were then manually homogenised in a 10cm dish withing 9mL of media (200 U/ml DNase, Cat:D-4527, Sigma-Aldrich, in 9 ml Dulbecco's modified Eagle's medium, DMEM). The medium containing the homogenized lung was then filtrated through 100, 40 µm (BD Biosciences), and 0.22 µm (EMD Millipore) strainers and centrifuged at 200g for 10minutes @4°C. The supernatant was subsequently discarded and the cells were resuspended in MACs buffer (MACS buffer: 500ml PBS containing 0.5% bovine serum albumin, 130–091–376, Miltenyi Biotec and 2 mM EDTA, AM9260G, Invitrogen). The resuspended cells were then sorted through magnetic selection for CD45 negative and subsequently EPCAM positive (CD45-EPCAM+) cell population. Once isolated cells were washed with PBS and pelleted @1500xg for 5minutes. Isolated CD45-EPCAM+ cells were then either resuspended in AT2 medium for seeding, Trizol for RNA extraction, or RIPA lysis buffer for protein quantification of GF-AAS measurements.

2.3.3.2 Bronchoalveolar lavage fluid retrieval

BALF was collected from euthanized mice by lavaging the lungs through a cannulated trachea with 700 µL increments of ice-cold PBS. The initial 700 µL was centrifuged at 450g for 5 minutes, and the supernatant was retained for cytokine, protein, surface tension, GF-AAS, heme and porphyrin quantification. Subsequent lavages (2nd and 3rd) used PBS with 0.5 mM EDTA and cells were pelleted under the same conditions. Cell pellets from all three lavages were pooled for cell counts and population analysis via cytopins. Given compromised lung integrity in diseased mice, variations in PBS recovery were noted; thus, volumes retrieved were recorded and downstream analyses were normalized to the total PBS recovered per mouse. Protein levels in the BALF was measured by BCA as described above. For GFAAS measurements or iron in BALF equal parts of 50% nitric acid was added to digest the sample, as described above.

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2.3.3.3 Cytospin preparation

Pelleted bronchoalveolar lavage (BAL) cells from the initial 2.1 mL of BAL fluid (BALF) were resuspended in 1 mL of AKT red blood cell (RBC) lysis buffer and incubated for 1 minute at room temperature. The cell suspension was then centrifuged at 450xg for 5 minutes. Additional RBC lysis buffer treatments were performed as needed if the pellet exhibited a red appearance. The resulting pellet was resuspended in 225 μ L of phosphate-buffered saline (PBS), and a 20 μ L aliquot was removed for cell quantification using a hemacytometer. For cytopsin preparation, 100 μ L of the cell suspension was loaded and processed using a Shandon Cytospin 3 at 350xg for 5 minutes. The cytopsin slides were stained using the Kwik-Diff differential stain, as per manufacturer's instructions and scanned at a 20X objective. The differential cell count was performed by counting a total of 300 cells on each slide and the percentages of macrophages, lymphocytes and polymorphonuclear leukocytes (PMNs) were calculated, utilising ImageJ cell counter software. The absolute cell counts for each cell subset were then calculated.

2.3.3.4 Tissue digestion and preparation

Tissue samples were placed in cryovials, flash-frozen in liquid nitrogen, and stored at -80°C. Using a blade, approximately 5 mg sections were carefully cut while keeping samples on ice to prevent thawing; for lung tissues, small pieces were taken from the apical, middle, and alternating lobes. Samples were then rinsed with ice-cold PBS containing protease inhibitors. Samples were repeatedly washed until tissue appeared less red. For lysis, 300 μ L of RIPA buffer was added, with volumes adjusted relative to tissue mass. The tissue was homogenized in 15-second intervals, with the probe washed sequentially in water and ethanol between samples. Samples were then sonicated using a probe at 20 kHz (cycle 5, Level 5) for 5-10 seconds. The sonicator probe was cleaned between samples using water and 70% ethanol to avoid cross-contamination. Finally, samples were centrifuged at 12,000xg for 20 minutes at 4°C, with the supernatant carefully collected and stored @-20°C before protein quantification and immunoblot or GFAAS analysis.

2.3.3.5 AT2 cell trans-differentiation into AT1 cell procedure

To initiate differentiation, 12-well plates were pre-coated with Collagen I (0.1 mg/mL) one day prior to plating. Primary murine AT2 cells (CD45⁻/EPCAM⁺) were isolated, washed, and resuspended in AT2 media (DMEM, 10% FBS, p/s, 10 mM HEPES). Cells were counted and plated at a density of 1–2 $\times 10^6$ cells/mL, pooling cells from at least three mice. Markers analysed included AT2 (Surfactant Protein C, *SftpC*), Transitional State (Keratin 8, *Krt8*), and

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AT1 (Aquaporin 5, *Aqp5*). Cells were collected at Day 0 (freshly isolated), Day 1 (24 hours post-media change), and subsequently every two days (Day 3, Day 5, and Day 7). On Day 0, control cells were pelleted immediately in Trizol. At 24 hours, adhered cells were refreshed with new media, and floating cells were pelleted, resuspended, and returned to their original wells. For harvesting, cells were scraped, pelleted, washed with PBS, and preserved in Trizol at -80°C for RNA extraction. The expected RNA yield was between 80–1600 ng.

2.3.3.6 Large-Scale lipidomics

Lipidomic data-sets were analysed as part of this project, sample collection was performed by Li-Chao Fan in Cornell, New York. Processed data was analysed by Sarah Kenny (me). Details on sample collection and data processing are provided in Fan *et al.* (2023) (209). Briefly, lipids were extracted from isolated AT2 cells, lung tissue and BALF by a chloroform–methanol extraction method. Samples were analysed using a 6490 Triple Quadrupole LC/MS system (Agilent Technologies) spiked with appropriate internal standards (209).

2.3.3.7 RNA-Seq analysis in isolated AT2 cells

RNA samples were obtained from isolated AT2 cells from *Mfrn1*^{+/-}*Mfrn2*^{iAT2} and *Sftpc*^{CreERT2+/-} mice. RNA was extracted using TRizol and purified with the RNeasy Plus Mini Kit, as detailed by manufacturer instructions. The RNA integrity number (RIN) and quality (A260/280) of the isolated RNA samples was quantified before sequencing via HiSeq4000 sequencer (Illumina). Libraries were constructed using the TruSeq Stranded mRNA library Preparation kit (Illumina), according to manufacturer's instructions. Of note, only high quality RNA with a 260/280 ratio larger than 1.6 and a RIN > 7 was used for library construction. The cBot fluidic device (Illumina) was used to hybridize samples onto a flow cell and to generate clonal clusters of the DNA fragments. Reads were then aligned and mapped to the mouse reference genome by TopHat2 (version 2.0.11) (<https://ccb.jhu.edu/software/tophat/index.shtml>)(218) , and transcriptome reconstruction was performed by Cufflinks (version 2.1.1) (<https://cole-trapnellab.github.io/cufflinks/>). Transcript abundance was measured with Cufflinks in Fragments Per Kilobase of exon model per Million mapped reads (FPKM) (219, 220). Utilizing the DESeq2 package (<https://bioconductor.org/packages/release/bioc/html/DESeq2.html>) (221) expression profiles were constructed for differential expression analyses. To assess the differential gene expression, adjusted p-values for multiple testing were calculated via the Benjamini-Hochberg method. Specific GO terms related to biological processes, including iron homeostasis, cellular senescence and surfactant metabolism, were searched and utilized to investigate significantly differentially expressed genes in these categories. These genes were

then visualized on heatmaps. Heatmaps were plotted using heatmapper.ca (1), based on the corresponding z-score calculated via gene expression by FPKM.

2.4 Statistical Analysis

All statistical analysis was performed utilizing GraphPad Prism software (Version 9.3.1). For data sets with two experimental groups (i.e. treated or untreated), unpaired students T-test (*) were utilized to test for statistical significance. For *in vitro* experiments, unpaired t-tests were used between biological replicates, where each experimental 'n' was treated as an independent group, as cells were seeded, treated, and analysed on separate days. Paired t-tests were used for technical replicates, as stated clearly in each figure legend. For *in vivo* experiments, unpaired t-tests were used between groups, with each mouse representing an individual biological replicate. Statistical significance for three or more groups were assessed via one- or two-way analysis of variance (ANOVA) (#) followed by Tukey's correction. Dunnett's test were carried out after ANOVA analysis for multiple treatment groups with one single control, as detailed in figure legends. Statistical significance was recorded at the following P-values; #/* $p < 0.05$, ##/** $p < 0.01$, ###/*** $p < 0.001$, ####/**** $p < 0.0001$, as detailed in figure legends. For volcano plots, threshold cut-off points included adjusted p-value < 0.05 , log2fold change > 2 . Of note, if visible outliers were identified during analysis the ROUT method for identifying and removing outliers was applied. The false discovery rate (Q value) was set at 1% to maintain a conservative approach, ensuring a low probability of incorrectly designating genuine data points as outliers. This stringent threshold was chosen to minimize the impact of false positives, thereby preserving the integrity of data analysis.

3. The AT2 Cell Tool-Kit

3.1 Introduction

Since the first isolation of AT2 cells in 1974, our understanding of these multi-functional cells has advanced significantly (4). However, their study increasingly relies on complex and costly techniques, limiting accessibility. AT2 cells perform a variety of critical functions in the alveoli, including (a) surfactant maintenance, (b) alveolar epithelial repair, (c) innate immunity, (d) fluid regulation, and (e) alveolar homeostasis (**Table 3.1**). Research in the field often centres on a single aspect within one of these function, making it difficult to interpret how accurate the model recapitulates the specialised functions of these cells. In light of this, this chapter focuses on the two primary functions of AT2 cells - surfactant maintenance and reparative capacity – exploring each node within these functions and evaluating current available readout and their applications to existing models, in an attempt to identify gaps in these models when studying AT2 cells.

3.1.1 Surfactant Homeostasis

Surfactant homeostasis involves the synthesis, secretion, and recycling of pulmonary surfactant. Lipidomic analysis via liquid chromatography-mass spectrometry (LC-MS) is one of the most robust methods for analysing surfactant composition, offering insights into lipid regulation and synthesis in AT2 cells or secreted surfactant from BALF (209). However, lipidomics is costly and inaccessible for many researchers, who therefore often rely on immunohistochemistry or gene expression analysis of surfactant proteins (222). These approaches are also limited, as surfactant proteins constitute less than 10% of surfactant, and precursor protein analysis (e.g. pro-SFTP-C) provides little insight on the processed functional protein (3).

Assessing surfactant turnover is challenging. Early methods used radioactive PC, but its short half-life and altered trafficking are drawbacks (24, 223). Recent advancements, such as Click-Chemistry, have facilitated the fluorescent labelling of choline-containing phospholipids (CPLs), enabling improved visualization of PC and sphingomyelin (SM) trafficking. However, the use of bolus dosing, the administration of a large and concentrated dose, presents challenges as to where exactly the signal is coming from as CPLs are present throughout the cell and not just within surfactant (224). Both methods provide indirect assessments of surfactant, as PCs also play broader cellular roles.

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Surfactant biophysical properties, critical for surface tension lowering function, are measured using techniques such as captive bubble and pendant drop methods. The captive bubble method best simulates breathing mechanics, assessing both maximum and minimum surface tension, while the pendant drop method measures maximum capacity with control of environmental factors (225). Each method offers complimentary insights into surfactant performance under physiological conditions.

While much of the literature focuses on specific aspects of surfactant regulation, a more comprehensive approach would be to examine surfactant composition, secretion, turnover, and biophysical properties together. This would provide a clearer homogenous picture of how AT2 cells maintain surfactant homeostasis in both health and disease.

3.1.2 Repair and Regeneration

In addition to their role in surfactant maintenance, AT2 cells are crucial for lung repair, particularly through their ability to transdifferentiate into AT1 cells and self-renew to restore the alveolar epithelium after injury (5, 6). Advanced lineage tracing, combined with single-cell analysis, reveals cellular populations and pathways involved in lung regeneration, often using murine models of epithelial injury (5, 75, 226). However, in lung diseases, AT2 cells typically enter a dysfunctional state rather than being completely lost, limiting the relevance of direct AT2 injury models for studying repair mechanisms (75, 226). Organoid cultures can assess AT2 repair capacity but are limited by binary outcomes ("do or don't form organoids"), which fail to capture intermediate cell states. Recent studies partially address this limitation with the development of a novel human foetal lung-derived alveolar organoid model capable of differentiating into AT1-like cells. However, differentiation is achieved under AT1 lineage-promoting conditions, introducing potential bias and limiting the representation of the natural differentiation capacity of these cells, warranting further investigations (227). The integration of organoid cultures with single-cell analysis enables the capture of transitional cell states and offers valuable insights into AT2 self-renewal and trans-differentiation. However, these approaches are highly specialized, costly, and not always accessible.

Alternatively, the "wound-healing" assay offers a straightforward and economical way to evaluate AT2 cell repair (228). The assay involves creating a scratch/injury to a monolayer of cells and assessing wound closure overtime. While simple, it can be applied to both primary and immortalised cultures. Furthermore, microenvironmental factors (e.g. nutrients) can be manipulated in a controlled injury model. Though it provides limited mechanistic insights, when

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integrated with other methods like organoid cultures or single-cell analysis, the wound-healing assay complements the study of AT2 cell regeneration and differentiation.

Although AT2 cells also play a key role in innate immunity and fluid regulation these aspects are beyond the scope of this thesis. This study specifically focuses on surfactant homeostasis and reparative capacity, as impairment of these functions have been implicated in several chronic lung diseases (8).

Table 3.1: Functional readouts of AT2 cells

Function of AT2 Cell	Description	Current Readouts	References
(a) Produce, secrete and turnover pulmonary surfactant	AT2 synthesize and secrete pulmonary surfactant, a lipid rich compound that regulates surface tension in the alveoli. AT2's account for ~65% of surfactant recycling.	Large Scale Lipidomic Analysis Click-IT Chemistry Radio-labelled Phosphatidyl Choline Immunohistochemistry of surfactant proteins Gene Expression analysis of surfactant genes Surface Tension Analysis	(3, 24, 223-225, 229, 230)
(b) Alveolar Epithelial Repair	Trans-differentiation into AT1 cells - AT2 cells act as the progenitors for AT1 cells Self-renewal: AT2 cells can divide and replace themselves	Wound healing assay Gene Expression analysis of signalling pathways associated with repair Cell-labelling and lineage tracking Organoid Culture	(5, 228, 231)
(c) Immunogenic Capacity	AT2 cells serve as a barrier to invading pathogens and secrete chemokines/cytokines to boost host defence by activating neighbouring immune cells.	Analysis of antigen presentation and cytokine production Surfactant Proteins (SftpA and SftpD)	(4)
(d) Regulation of Alveolar Fluid	Air-Blood Barrier: AT2 and AT1 cells create a functional barrier, preventing vascular	Trans-epithelial electrical resistance (TEER)	(4, 232)

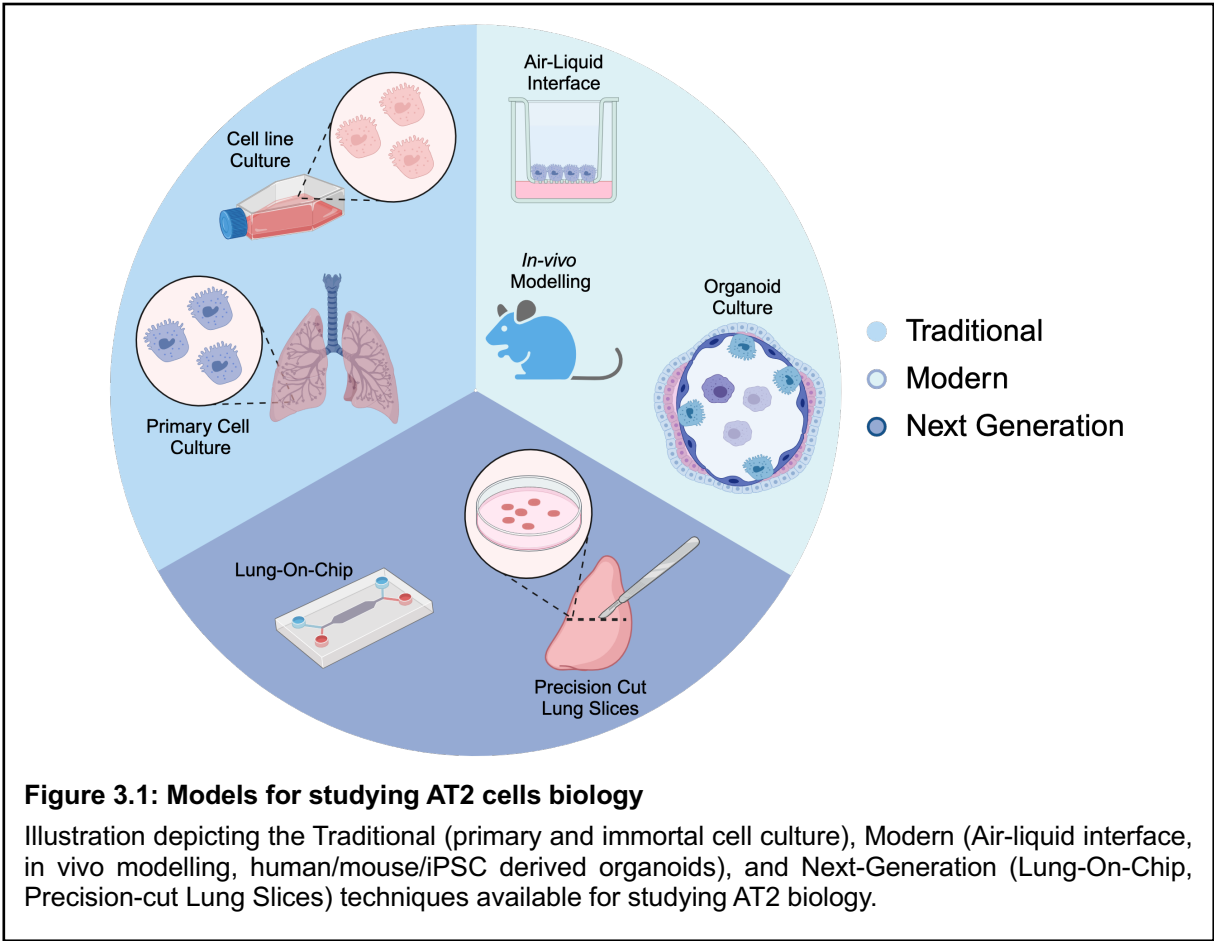
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Function of AT2 Cell	Description	Current Readouts	References
	leakage into the alveolar space.	Gene Expression analysis of aquaporins and ion channels	
(e) Regulatory Capacity	AT2 cells play a role in maintaining alveolar homeostasis by communicating with resident cells	Co-culture models to explore the cross-talk	(231, 233)

3.1.3 Limitations and Advancement of Current Models

To study AT2 cell function, a variety of experimental models have been developed, including *in vitro*, *in vivo*, and *ex vivo* systems, each offering distinct advantages and limitations depending on the specific research question (**Figure 3.1**).

Traditionally, AT2 cells were characterized using primary isolates from animal tissues, a method still widely used today (223, 234-236). However, advances in isolation techniques now



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enable purities exceeding 90%, achieved by enzymatically digesting lung tissue and selecting for surface markers such as EpCAM and MHCII via magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) (81, 209, 231, 237-239). Notwithstanding, the isolation and use of primary AT2 cells presents with several limitations, including rapid transdifferentiation into AT1 cells when cultured *ex vivo*, slow proliferation, limited expansion capacity, and poor cryopreservation potential (238, 239).

Due to these challenges, many researchers turn to immortalized AT2 cell lines as more practical and reproducible alternatives. Additionally, ethical considerations surrounding the use of animals in research, guided by the principles of the 3Rs (Replacement, Reduction, and Refinement), have also encouraged a shift toward *in vitro* models.

Immortal cell lines, such as human A549 and H441 and murine MLE-12 and MLE-15, are widely used for modelling AT2 cells (240). A549 cells are cancerous in origin, and although commonly used and capable of expressing surfactant proteins, A549 resemble AT1 cells in phospholipid composition and lack tight junction formation, limiting their suitability for permeability studies (241, 242). H441 cells, forming polarized monolayers at air-liquid interfaces, better model alveolar tightness and are useful for drug delivery research (222, 240, 243). However, they are characterized as a heterogeneous population with features of both AT2 and AT1 cells.

MLE-12 and MLE-15 cells, derived from transgenic mice harbouring the viral oncogene simian virus 40 large tumour antigen under the transcriptional control of the *Sftpc* promoter, express surfactant proteins and phospholipids, with MLE-12 cells showing stronger AT2-like features (244). Although these cell lines display varying degrees of AT2 characteristics, they often lack key features of native AT2 cells. Their ability to secrete and uptake surfactant lipids and proteins with the necessary biophysical properties to reduce surface tension have yet to be characterized.

Efforts to overcome limitations of traditional AT2 cell models have led to advancements in culture techniques including the development of alveolar organoid cultures. Derived from primary AT2-enriched murine or human lung cells, alveolar organoids can retain AT2 characteristics for months with specific growth media and feeder cells (226, 231). However, their reliance on freshly isolated primary cells limits accessibility and reproducibility.

Induced pluripotent stem cells (iPSCs) offer an attractive alternative to this issue. Reprogrammed from peripheral blood mononuclear cells, iPSCs can be differentiated into self-renewing AT2-like cells, forming epithelial-only spheres (iAT2) that can be maintained in

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culture for up to a year and model patient-specific lung mutations (245, 246). Recent advancements have led to the development of a human foetal-derived alveolar organoid model capable of secreting mature surfactant proteins, representing a promising step forward (227). However, further investigation are required to determine whether these organoids also secrete surfactant lipids. Additionally, it remains unclear if the secreted material from these organoids recapitulates the biophysical properties of native surfactant. Despite their promise, iPSCs require lengthy protocols, specialized media, and marker-based sorting, and their ability to fully replicate key AT2 functions, such as surfactant secretion and turnover, has yet to be established.

While traditional and modern models of AT2 cells have their respective advantages, they both fall short of fully recapitulating the physiological relevance of native AT2 cells. To overcome these limitations, advanced Lung-on-Chip systems have been developed to more accurately mimic the alveolar microenvironment. Lung-on-Chip models recreate key aspects of the alveolar microenvironment, including 3D micro-curved membranes, vascular flow, air-liquid interfaces, cyclic breathing motions, and multicellular interactions (27, 228, 231). They have been used to study AT2 cell differentiation, function, and regeneration, as well as innate immune responses, revealing a protective role of mechanical breathing in immunity (27). The platform supports integration of various cell types, including patient-derived AT2 cells expanded through organoid culture (27, 228, 231).

While *Lung-on-Chip* models offer significant potential for studying AT2 biology under realistic conditions, their high cost and technical complexity currently limit accessibility. Furthermore, whether or not AT2 cells carry out normal surfactant functions in this model remains unknown.

3.1.4 Chapter Aims and Objectives

A key challenge in AT2 cell biology is the lack of a standardized and comprehensive approaches for studying their functions. Most research focuses on isolated aspects of AT2 cell function, often investigating a single facet within a specific context without fully characterizing whether the chosen model accurately recapitulates AT2 cell-specific functions. In this chapter, I aim to address these challenges by evaluating the strengths and limitations of current techniques, while developing an economical accessible, **AT2-cell tool-kit** for *in vitro* and *in vivo* studies. By promoting a multimodal approach, the **goal** of this chapter is to provide more reliable and cost-effective tools that will advance our understanding of AT2 cell biology in lung health and disease.

This chapter aims to;

1. Develop and delineate distinct cell-specific functional readouts for AT2 cells.
2. Establish an appropriate model of AT2 cells *in vitro*.
3. Explore additional models to study AT2 cell function *in vivo*.

3.2 Results

3.2.1 MLE-12 Cells Are a Suitable *in vitro* Model for AT2 Surfactant Functional Studies

As mentioned, AT2 cells are primarily responsible for the production, secretion, and recycling of pulmonary surfactant. Approximately 80% of lipids found in surfactant consist of PL, with PC species constituting the main fraction. Beyond large-scale lipidomics, which is expensive and inaccessible (as previously highlighted), surfactant functionality in AT2 cells is often overlooked. Current functional assays lack sensitivity and specificity, particularly as they focus on surfactant proteins, which represent a minor component of surfactant. To address this, surfactant homeostasis was characterized into five functional aspects, and readily available assays were developed to provide a comprehensive overview of surfactant functionality. (**Figure 3.2A**). As primary AT2 cells are difficult to obtain and maintain in culture we opted for the murine MLE-12 cell line which has been previously shown to produce and regulate pulmonary surfactant components [1].

1. Biophysical Properties:

The primary function of surfactant is to reduce the surface tension at an air-liquid interface. Using a pendant drop tensiometer we analysed the surface tension in MLE-12 supernatant (supMLE-12), we see a significant decrease in surface tension when compared to the media control (**Figure 3.2B**).

2. Surfactant Turnover/Uptake:

To acquire the precursors for surfactant production, AT2 cells can either undergo *de novo* synthesis of FAs or uptake exogenously secreted surfactant and recycle the lipids (13). Focused analyses on DPPC, the most abundant phospholipid in the PC pool, revealed that despite AT2 cells constituting a small fraction of the alveolar wall, they are responsible for the majority of DPPC uptake (32, 247). A fluorescent NBD-DPPC uptake assay was used to indirectly assess surfactant uptake by AT2 cells. MLE-12 cells successfully demonstrate the uptake of NBD-DPPC (**Figure 3.2C and 3.2D**).

3. Surfactant Secretion:

We investigated surfactant secretion in MLE-12 cells by stimulating them with a secretagogue known to induce exocytosis in primary AT2 cells (24, 248). Phorbol 12-myristate 13-acetate (PMA), a potent phorbol ester, triggers surfactant secretion via

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protein kinase C activation and Ca^{2+} uptake (24). A PC assay kit was used to indirectly assess surfactant secretion and composition. Consistent with prior findings, we show that MLE-12 cells secrete phosphatidylcholine upon PMA stimulation (**Figure 3.2E**) (244). SupMLE-12 after PMA stimulation exhibited lower surface tension levels compared to the untreated control (**Figure 3.2F**). The PC assay results, combined with surface tension measurements, indicate that PMA stimulation promotes the secretion of surface tension-lowering phospholipids, consistent with functional surfactant activity.

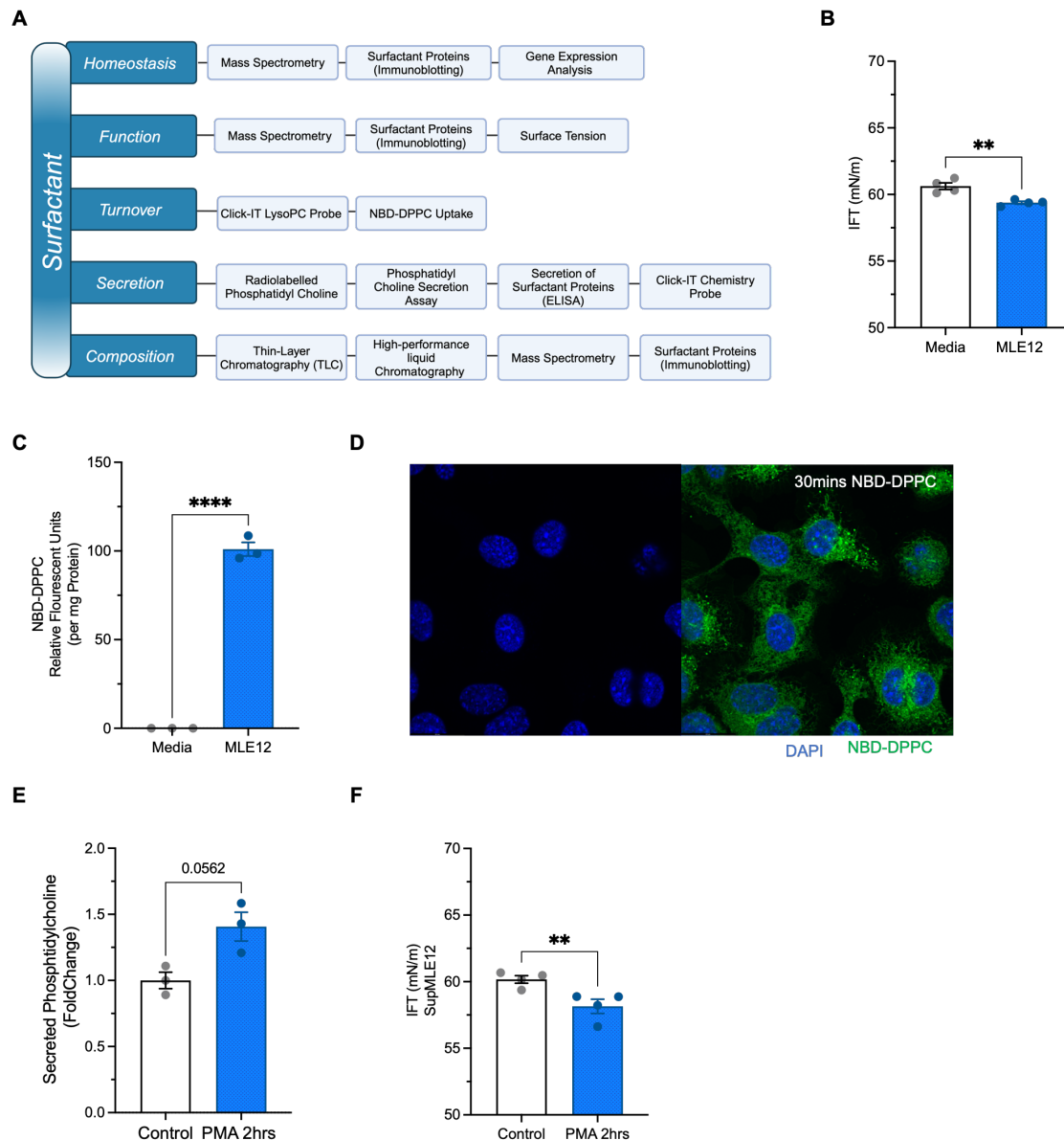
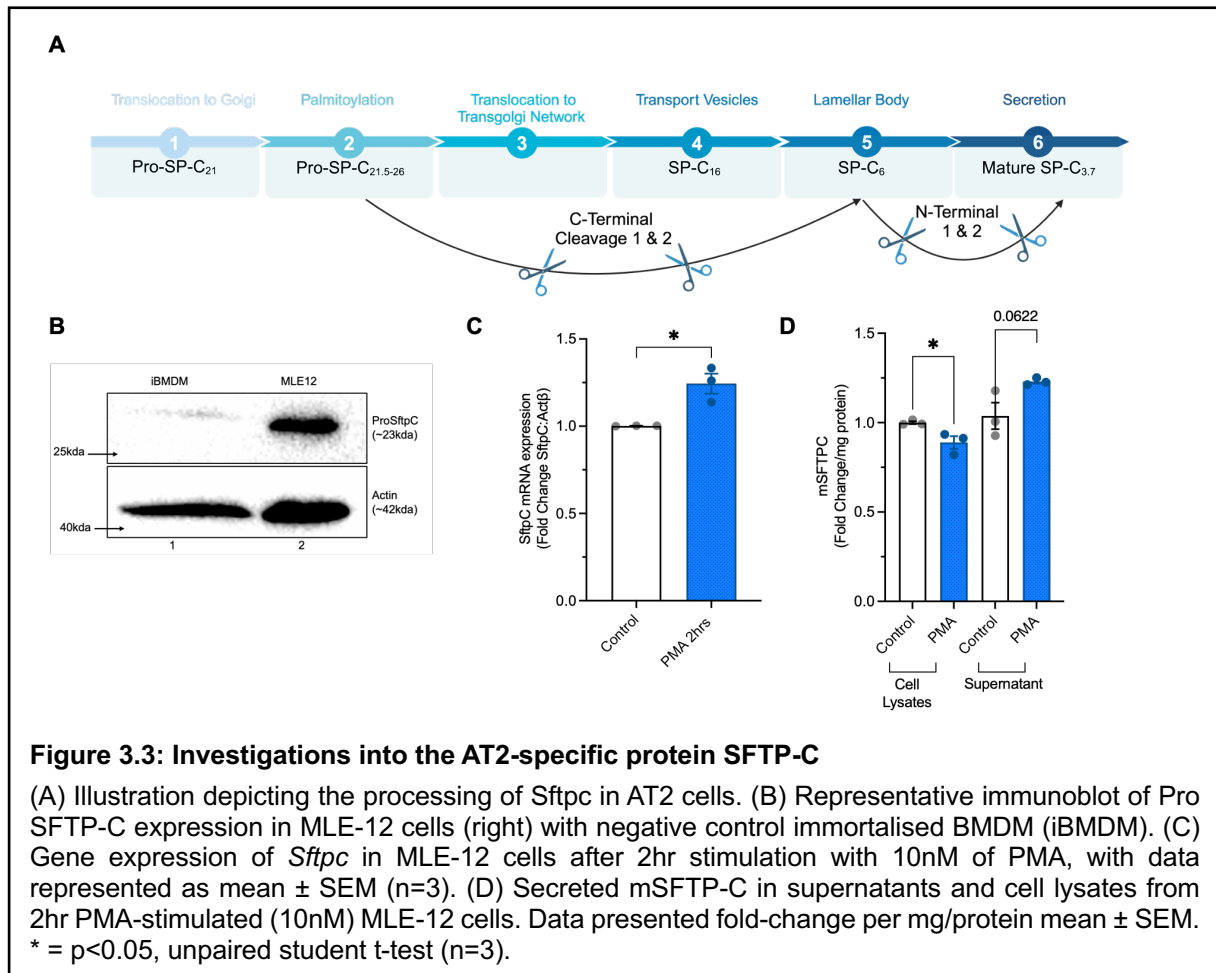


Figure 3.2: Comprehensive exploration of surfactant readouts in AT2 cells

(A) Illustration of techniques used to investigate surfactant composition, secretion, turnover, function and homeostasis. (B) Surface tension (IFT mN/m) in media alone and media from cultured MLE-12 cells measured with a sessile drop tensiometer. (C) Surfactant uptake assay in MLE-12 cells. Cells were exposed to 30 ng/ml of NBD-DPPC for two hours and fluorescence readings taken at 464 nm (ex) / 531 nm (em). (n= 3 technical replicates). (D) Representative image of confocal microscopy shows NBD-DPPC fluorescence (green) uptake in MLE-12 cells after 30minutes. Nuclei were counterstained with DAPI (blue) for reference. Scale bar = 100µm. (E) Alteration in PC levels in supernatant collected from PMA-stimulated (10nM) MLE-12 cells for 2hrs. Of note, fold-change was determined by comparing the initial supernatant (at 0 hrs pre-stimulation) to the subsequent increase in PC levels using a coupled enzyme assay. (F) MLE-12 cells were stimulated with 10nM PMA for 2hrs. Supernatant was collected and surface tension was measured using a sessile drop. All data presented as mean ± SEM. P-value calculated by unpaired student t-test, ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, ns = non-significant. Each data point represents an individual biological replicate.

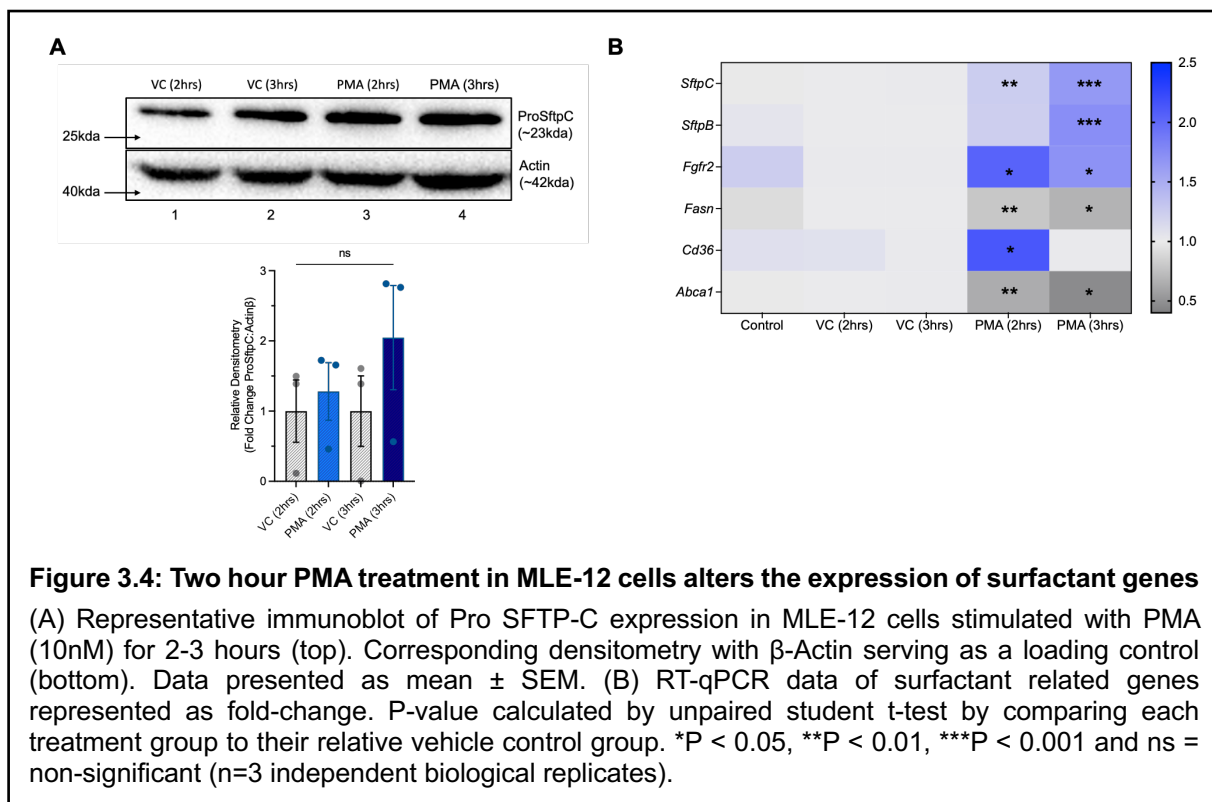
4. Surfactant Proteins:

SFTP-C expression is enriched in AT2 cells with studies suggesting the protein to be exclusively processed and secreted by AT2 cells (248). Analysing various forms of SFTP-C (pro, secreted, or transcript) provides broad insights into surfactant function (**Figure 3.3A**). Immunoblotting reveals that Pro-SFTP-C is expressed solely in MLE-12 cells when compared to an immortalised bone marrow derived macrophage (iBMDM) cell line (**Figure 3.3B**). PMA stimulation modestly increases *Sftp-c* gene expression (**Figure 3.3C**), couple with trends towards increased secretion of mature SFTP-C (mSFTP-C) (**Figure 3.3D**) and intracellular Pro-SFTP-C levels (**Figure 3.4A**). Here, intracellular levels of mSFTP-C are significantly reduced (**Figure 3.3D**), potentially driving a compensatory upregulation in the expression of its pro-protein form (**Figure 3.4A**). Taken together these results highlight how multimodal analysis of SFTP-C can provide a more comprehensive view of the cell's functional state.



5. Surfactant related gene expression:

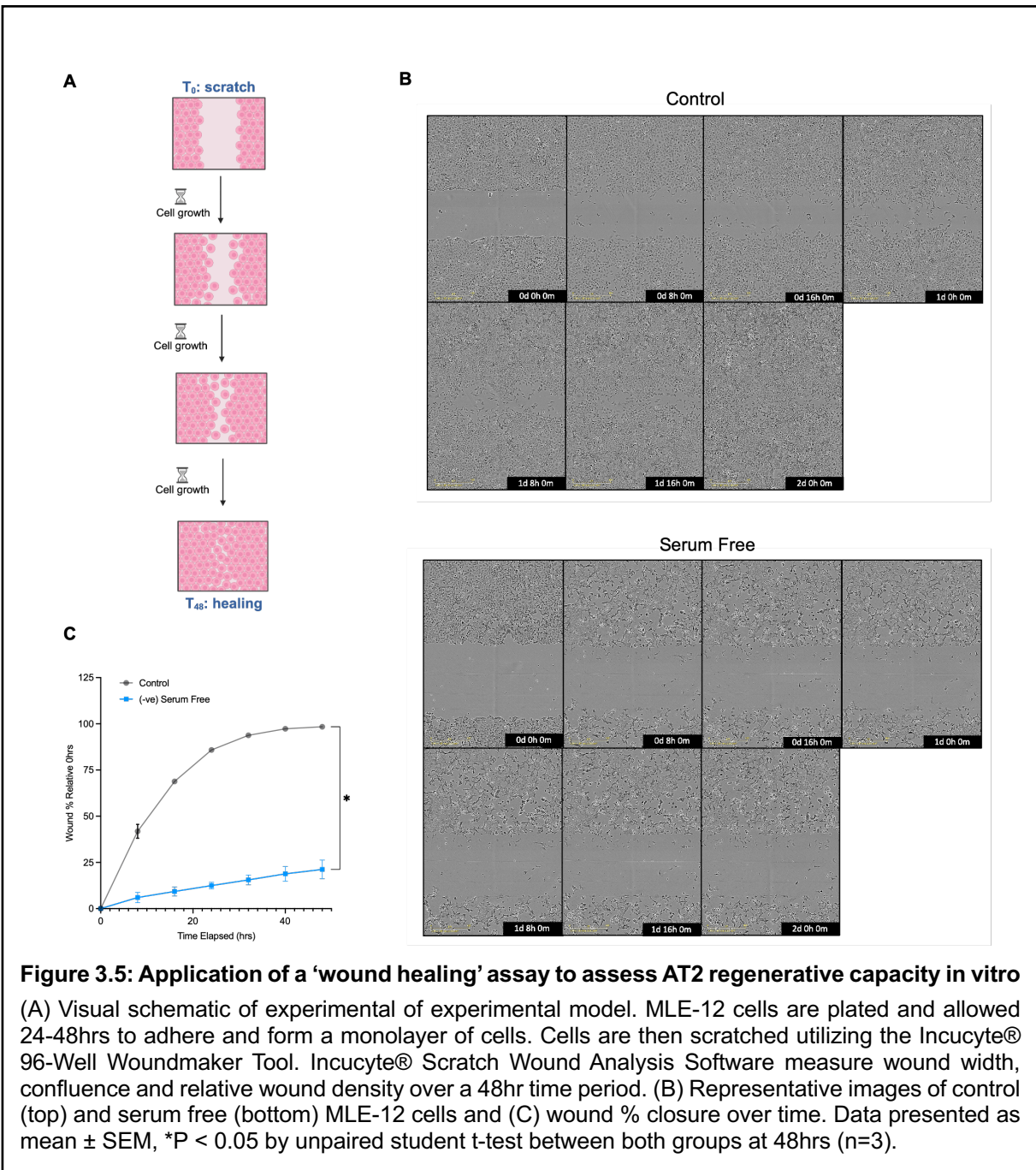
Surfactant function can be further assessed by examining genes linked to surfactant homeostasis. Including, *Cd36* (involved in the uptake of long-chain fatty acids (249)) and *Abca1* (facilitates cholesterol and phospholipid efflux (250)) for lipid transport; *Sftp-b* and *Sftp-c* (hydrophobic surfactant proteins essential for reducing surface tension), *Fasn* (critical for *de novo* fatty acid synthesis (251)), and *Fgfr2* (FGFR2 - mediated surfactant regulation (252)) for surfactant synthesis. After 2 hours of PMA stimulation, *Sftp-c* is upregulated, along with a significant two-fold increase in both *Cd36* and *Fgfr2*, suggesting lipid uptake and surfactant regulation (**Figure 3.4B**). Increased surfactant protein and transcript expression at 2-hours likely compensates for secretion-associated losses, supported by reduced intracellular mSFTP-C and increased secretion (**Figure 3.3D**). By 3 hours, *Sftp-c* and *Sftp-b* are upregulated, while *Cd36* expression returns to baseline (**Figure 3.4B**). The downregulation of *Abca1* may reflect reduced lipid efflux, and decreased *Fasn* suggests limited *de novo* lipid synthesis (**Figure 3.4B**). Further protein-level analysis is needed to confirm these findings.



These results indicate that MLE-12 cells contain the machinery to regulate surfactant homeostasis and can actively secrete and uptake surfactant, underscoring their relevance as an *in vitro* AT2 cell model.

3.2.2 Evaluating AT2 Repair Capacity Using in Vitro Models

Another defining characteristic of AT2 cells is their capacity to repair the epithelium upon injury, via trans-differentiation to AT1 cells and/or the ability to self-renew. To assess MLE-12 cells self-renewal capacity indirectly we employed an *in vitro* wound assay, where a 'scratch injury' was introduced and 'wound closure' monitored over time (**Figure 3.5A**). Under normal conditions, MLE-12 cells successfully migrated and closed the wound within 48 hours (**Figure 3.5B**). As MLE-12 cells are terminally differentiated and only 'AT2-like' in nature assessing their ability to transdifferentiate into AT1-like cells was not possible.



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3.2.3 Isolation and Characterization of Alveolar Type II Epithelial Cells from Mice

Primary AT2 cell isolation from mice is possible, by enriching for a CD45⁻ EPCAM⁺ cell population via MACs separation (**Figure 3.6A**). This population is enriched for AT2 cells and highly expresses Pro-SFTP-C when compared to the CD45⁺ isolated cell population (**Figure 3.6B**). This method allows for a ~90% AT2 cell purity of isolation (209). Once isolated, AT2 cells lose their AT2-like characteristics under normal culture conditions *ex vivo* and transdifferentiate into AT1 cells (238). Therefore, analysis on AT2 cells is primarily performed directly after isolation.

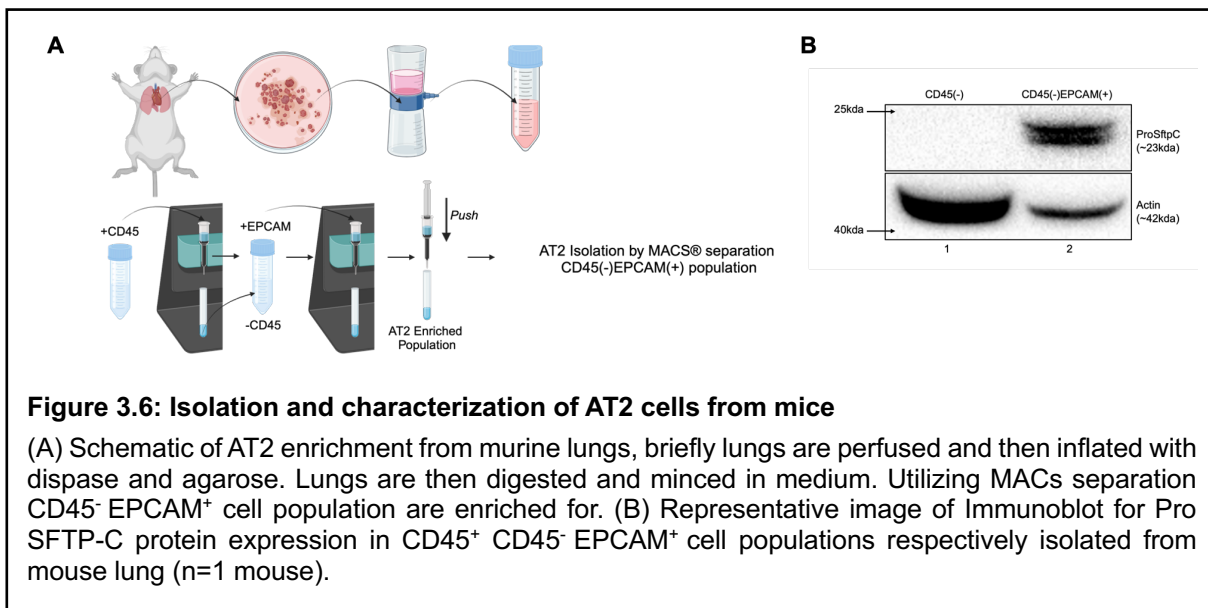


Figure 3.6: Isolation and characterization of AT2 cells from mice

(A) Schematic of AT2 enrichment from murine lungs, briefly lungs are perfused and then inflated with dispase and agarose. Lungs are then digested and minced in medium. Utilizing MACs separation CD45⁻ EPCAM⁺ cell population are enriched for. (B) Representative image of Immunoblot for Pro SFTP-C protein expression in CD45⁺ CD45⁻ EPCAM⁺ cell populations respectively isolated from mouse lung (n=1 mouse).

3.2.3.1 High throughput lipidomic analysis in healthy versus injured lung

When investigating surfactant homeostasis large-scale lipidomic analysis via liquid chromatography/mass spectrometry (LC/MS) on isolated AT2 cells can provide a comprehensive snapshot of AT2 surfactant activity *in vivo*. To characterize the lipidome of isolated murine primary AT2 cells, an existing dataset available in the lab (generated by Li-Chao Fan⁶) was analysed to assess surfactant-related lipids and their precursors. A previous publication on this work, to which I contributed through primary data analysis, confirmed that PL and free cholesterol (FC) were the most abundant lipid classes in AT2 cells from healthy mice, followed by sphingolipids and glycerolipids (209), consistent with literature (248). The available datasets also included lipidomics from AT2 cells of CS-exposed mice, a well-established COPD murine model. Briefly, mice were exposed to cigarette smoke (CS) for 2 hours daily, 5 days a week, for 6 months; subsequently, their AT2 cells were isolated for lipidomic analysis (see methods, **section 2.3.2 Chapter 2**).

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Here, our analysis revealed distinct differences in lipid profiles of AT2 cells from mice exposed to cigarette smoke for six months (**Figure 3.7A–3.7D**). While saturated fatty acids (SFAs) remained unchanged, monounsaturated fatty acids (MUFAs) showed an upward trend, and polyunsaturated fatty acids (PUFAs) were significantly increased in smoke-exposed AT2 cells compared to controls (**Figure 3.7A–3.7C**). Further heatmap analysis of significantly altered lipid species indicated that AT2 cells from smoke-exposed mice exhibit elevated levels of several lipids, which may have important implications for the surfactant phospholipidome in AT2 cells (**Figure 3.7D**). Consistent with previous findings, AT2 cells from CS-exposed mice showed an increase in total lipid levels, although the relative abundance of key surfactant-lipids were significantly reduced when compared to room-air (RA) controls (209). Notably, several SM and ceramide (CER) species were increased in isolated AT2 cells from smoked mice, highlighted in bold (**Figure 3.7D**). These SM and CER families have previously been reported to be elevated in the lungs of individuals with COPD (253, 254).

These findings underscore the power of lipidomics in uncovering altered AT2 cell function in murine disease models, offering valuable insights into the molecular mechanisms of COPD.

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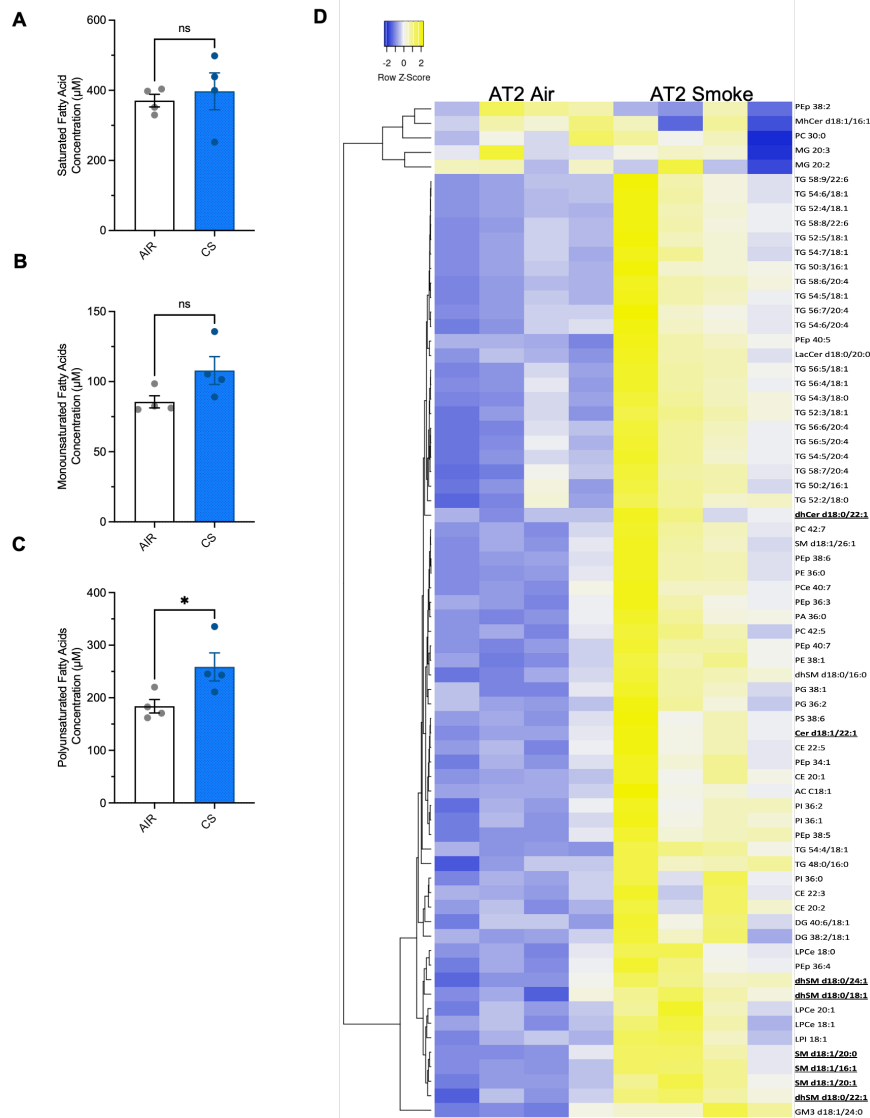


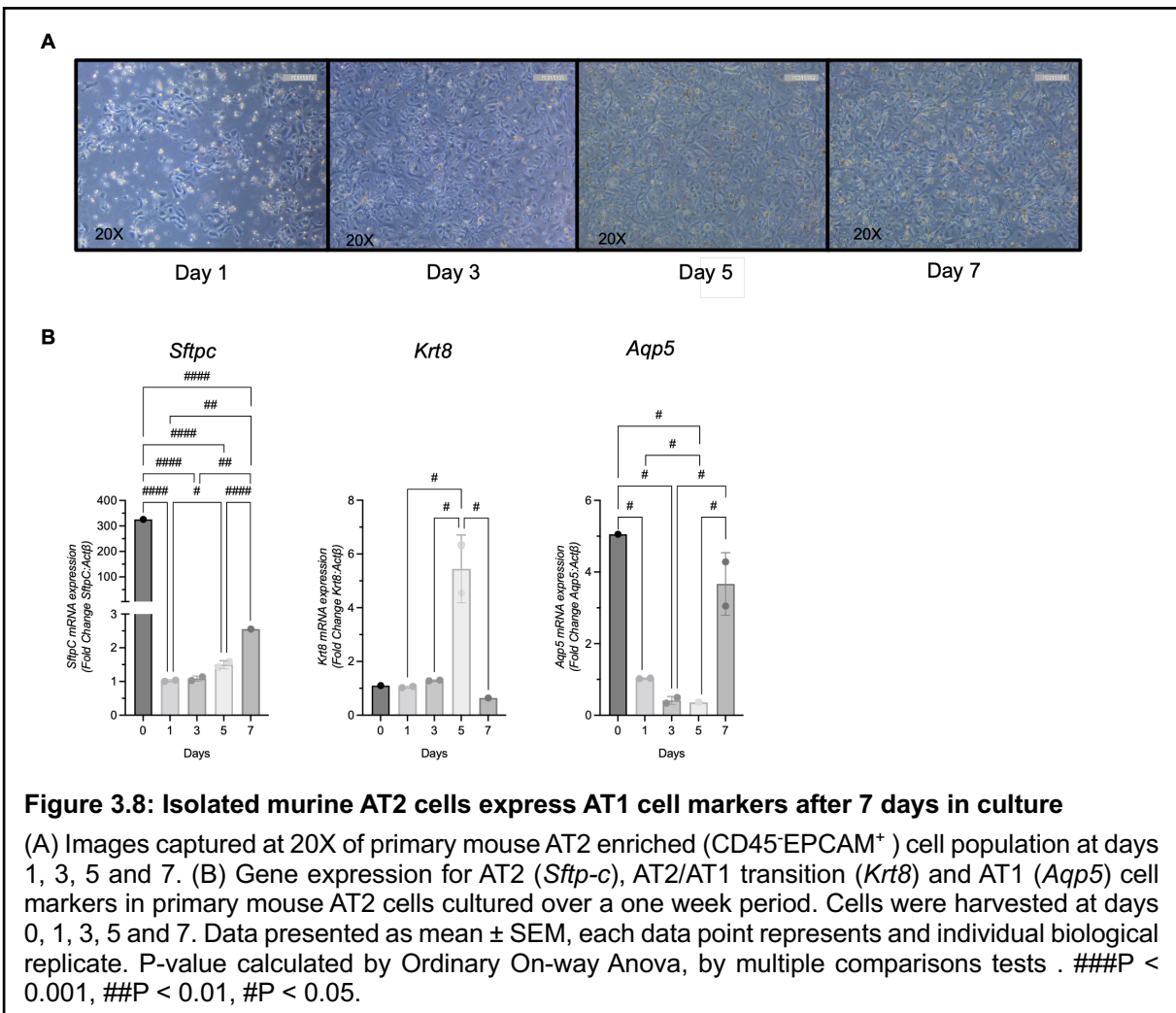
Figure 3.7: AT2 cells from CS-exposed mice present with an altered lipidome[€]

Concentration of (A) saturated fatty acids (B), monounsaturated fatty acids (C) and polyunsaturated fatty acids in AT2 cells isolated from mice smoked for 6 months and their retrospective controls. Each data point represents one mouse (n=4). P-value calculated by unpaired student's t-test to assess statistical significance between the smoked (CS) and untreated (Air) groups. *p<0.05, ns = non-significant. (D) Heatmap analysis, generated with heatmapper.ca, visualizes large-scale lipidomic analysis from isolated AT2 cells from RA-controls and CS-exposed mice for 6 months. Significantly upregulated (yellow) and downregulated (blue) lipid species in AT2 include FC (free cholesterol); CE (cholesterol ester); AC (acyl carnitine); MG (monoacylglycerol); DG (diacylglycerol); TG (triacylglycerol); Cer (ceramide); dhCer (dihydroceramide); MhCer (monohexosylceramide); Sulf (sulfatide); LacCer (lactosylceramide); GM3 (monosialodihexosylganglioside); GB3 (globotriaosylceramide); BMP (bis(monoacylglycerol)phosphate); AcylIPG (acyl phosphatidylglycerol); SM (sphingomyelin); PA (phosphatidic acid); PC (phosphatidylcholine); PCE (phosphatidylcholine ether); PE (phosphatidylethanolamine); PEP (plasmalogen phosphatidylethanolamine); PS (phosphatidylserine); PI (phosphatidylinositol); PG (phosphatidylglycerol); LPC (lysophosphatidylcholine); LPE (lysophosphatidylethanolamine); LPEP (plasmogen lysophosphatidylethanolamine); LPI (lysophosphatidylinositol); LPS (lysophosphatidylserine); NAPE (N-acyl phosphatidylethanolamine); and NAPS (N-acyl phosphatidylserine). Of note, underlined and bold species are implicated in COPD (n=4 mice per group).

€ - Where indicated, portions of this text and data were previously published, to which I contributed primary analysis and writing (please see declaration page i). Fan, L.C., et al., Alveolar type II epithelial cell FASN maintains lipid homeostasis in experimental COPD. JCI 133(11):2022-2032 (2023).

3.2.3.2 AT2 to AT1 cell differentiation

As mentioned, immortalized cell lines are unsuitable for assessing the transdifferentiation capacity of AT2 cells into AT1 cells (238, 239). To address this, we employed a transitional *ex vivo* model, isolating and culturing primary murine AT2 cells to assess the loss of AT2 markers overtime. AT2 cells were cultured on collagen-coated plates, with samples collected at Day 0 (freshly isolated). Cultured cells were then supplemented with fresh media the next day and samples collected at day 1 (24 hours post-media change), and subsequently every two days (Days 3, 5, and 7) (**Figure 3.8A**). Gene expression for AT2 (*Sftp-c*), AT1 (*Aqp5*), and AT2 to AT1 intermediate (*Krt8*) were analysed over time. *Sftp-c* expression decreased relative to Day 0 but showed an increase compared to Day 1 (**Figure 3.8B**). Transitional marker *Krt8* peaked at Day 5 with a <5-fold increase, indicating maximal trans-differentiation activity (**Figure 3.8B**). By Day 7, *Aqp5* expression significantly increased, marking terminal differentiation into AT1 cells (**Figure 3.8B**). These findings align with the literature, demonstrating that AT2 cells undergo peak trans-differentiation at Day 5, with terminal AT1 differentiation and marker expression by Day 7 (238, 239).



3.2.4 Exploiting the Lung-Specific SFTP-C Promoter for Targeted Gene Deletion to Assess AT2 Cell Function in Mice.

A common tool in the AT2 field is the use of the lung-specific SFTP-C promoter for targeted gene deletion in AT2 cells. *Sftp-c*^{CreERT2/+} mice (JAX stock #028054) crossed with loxP-flanked mice allow for tamoxifen-inducible gene deletion specifically in AT2 cells, providing insight into how gene loss affects AT2 function (see methods, **Section 2.3.9 Chapter 2**)

3.2.4.1 Disruption of surfactant synthesis in AT2 cells

As a proof of concept, we aimed to model disrupted surfactant synthesis in AT2 cells using available lab datasets and tools. Here we show by crossing *Fasn*^{loxP/loxP} homozygous mice with *Sftp-c*^{CreERT2/+} mice to generate *Fasn*^{loxP/loxP} *Sftp-c*^{CreERT2+/-} (*Fasn*^{ΔAT2}) mice we can successfully deplete the enzyme FASN protein levels in AT2 cells with tamoxifen (**Figure 3.9A and 3.9B**). FASN is a key regulator of lipid metabolism, enabling *de novo* synthesis of fatty acids (FAs) (251). Fatty acids serve as key precursors for the PLs in surfactant, and therefore FASN is crucial for surfactant production by AT2 cells (209, 251). Interestingly, FASN expression was enriched in AT2 cells when compared to whole lung highlighting the lipid-rich functions of AT2 cells (209).

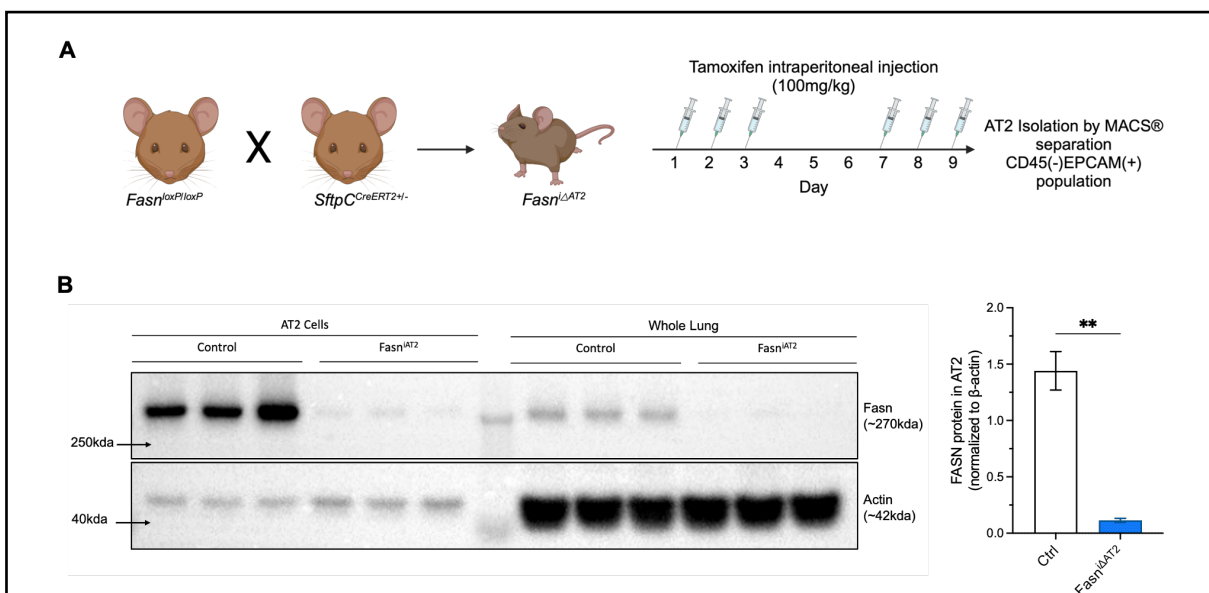


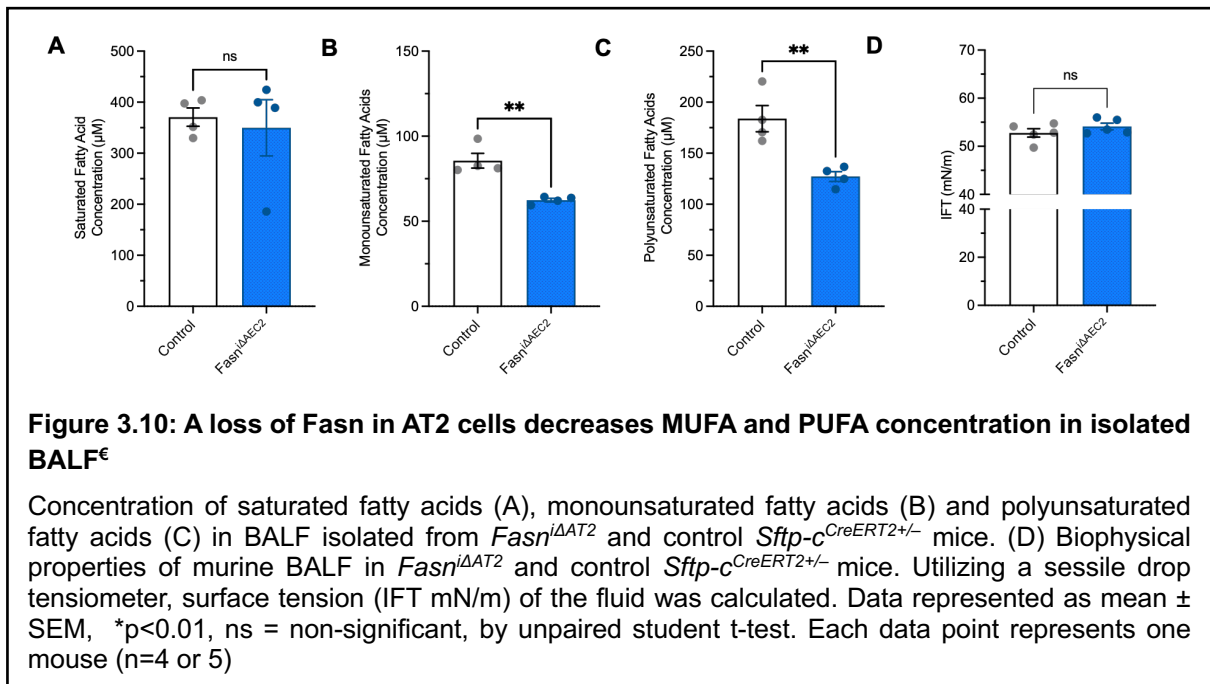
Figure 3.9: Exploiting the lung-specific SFTP-C promoter for targeted gene deletion to assess AT2 cell function[€]

(A) Schematic illustrating the generation of AT2-specific *Fasn*^{ΔAT2} mice through the cross-breeding of *Fasn*^{loxP/loxP} homozygous mice with *Sftp-c*^{CreERT2+/-} mice, utilizing the loxP-Cre tamoxifen-inducible system. (B) Representative immunoblot (left) with corresponding densitometry (right) depicting FASN expression in primary isolated AT2 cells and lung tissue from control *Sftp-c*^{CreERT2+/-} and *Fasn*^{ΔAT2} mice. β-Actin served as a loading control (n = 3 per group). **P < 0.01 by Student's unpaired t test.

€ - Where indicated, portions of this text and data were previously published, to which I contributed primary analysis and writing (please see declaration page i). Fan, L.C., et al., Alveolar type II epithelial cell FASN maintains lipid homeostasis in experimental COPD. JCI 133(11):2009-2120 (2013)

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Utilizing BALF, a diluted surfactant suspension, we can assess AT2-secreted surfactant function indirectly in *Fasn*^{ΔAEC2} mice and their respective controls. Lipidomic analysis via LC/MS of isolated BALF revealed a significant reduction in MUFA and PUFA species in *Fasn*^{ΔAEC2} mice (**Figure 3.10A and 3.10C**), while SFA levels remained unchanged (**Figure 3.10B**). To investigate this further, we used a pendant drop tensiometer and observed a slight trend towards increased surface tension, though no definitive conclusions could be drawn (**Figure 3.10A**). Together this suggests AT2-specific loss of *Fasn*, leads to decreased secretion of unsaturated lipid species into BALF from AT2 cells.



3.2.4.2 Smoke as an injury model in *Fasn*^{ΔAEC2} mice

Since minimal changes were observed at baseline, we explored the impact of *Fasn* loss in AT2 cells under experimental disease conditions. Mice were subjected to a CS-exposure model, with acute (6-week) and chronic (6-month) exposures, followed by collection of BALF and lung tissue for analysis. *Fasn*^{ΔAEC2} mice exposed to 6-weeks of CS showed a significant increase in surface tension compared to controls (**Figure 3.11A**).

Given that PC species constitute ~85% of the PL found in surfactant, we analysed the percentage of SFA, MUFA and PUFA PC species in isolated BALF. *Fasn*^{ΔAEC2} mice BALF had a notably lower abundance of SFA-PC lipids after smoke exposure compared to controls, while MUFA-PC levels remained unchanged (**Figure 3.11B**). Interestingly, there was a significant increase in PUFA-PC species in *Fasn*^{ΔAEC2} mice both at baseline and post-CS exposure when compared to their retrospective controls. This suggests an imbalance in SFA-PC, potentially promoting complex PUFA-PC species formation, contributing to increased surface tension and

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exacerbating AT2 cell response to smoke. Further analysis of altered PUFA phospholipid species in BALF from smoke-exposed *Fasn*^{iΔAEC2} mice revealed PUFA-PC species as the most commonly increased lipid group, as shown by volcano plot analysis (**Figure 3.11C**). These findings indicate an overall increase in PUFA species in *Fasn*^{iΔAEC2} mice, particularly after smoke exposure.

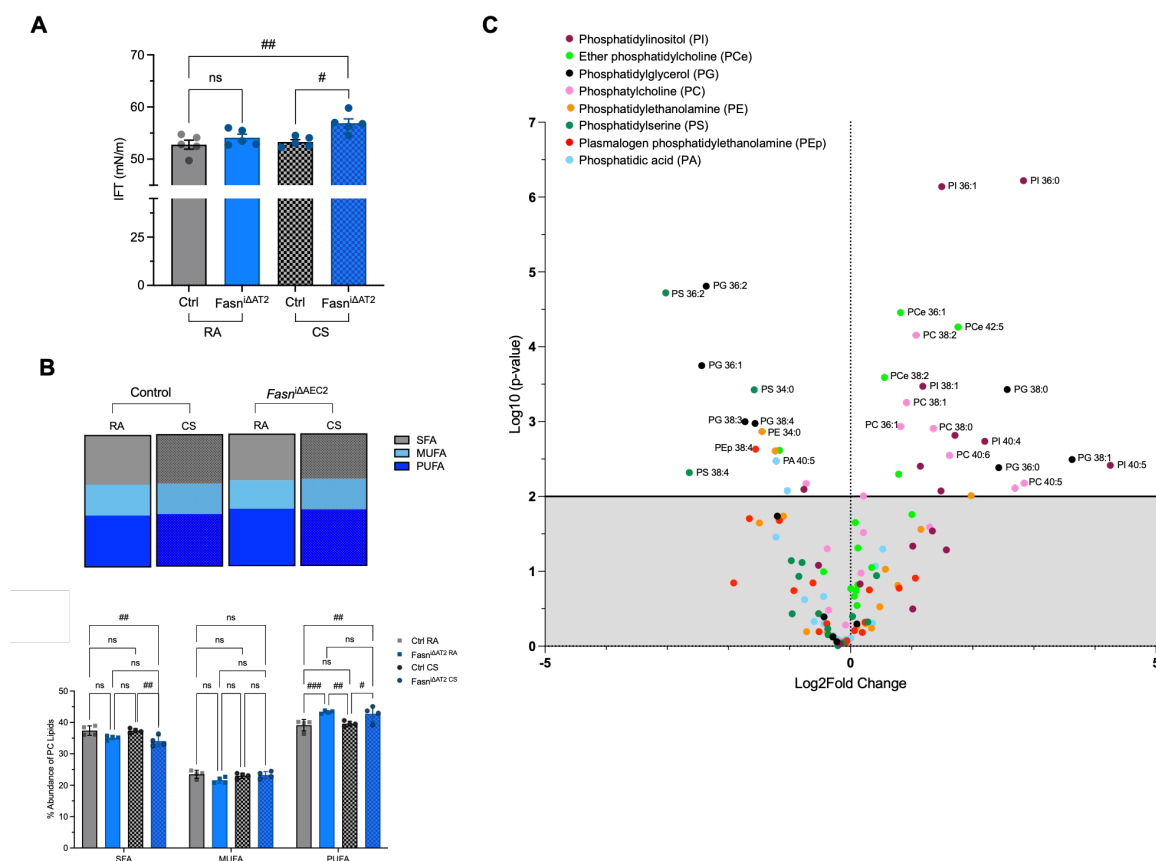
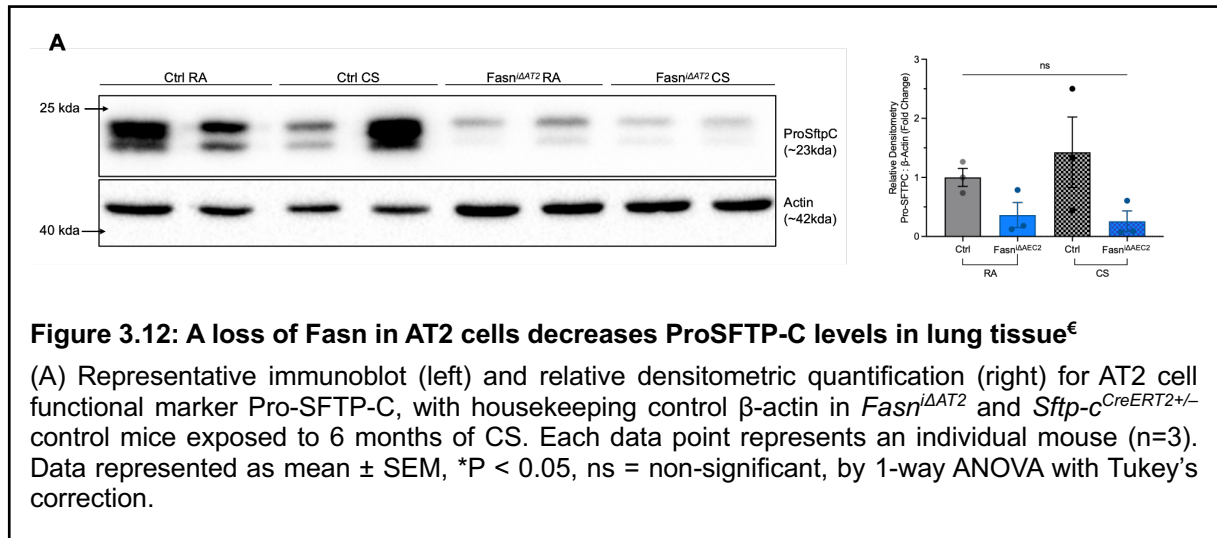


Figure 3.11: A loss of Fasn in AT2 cells increases surface tension and PL-PUFA species in BALF after acute smoke exposure[€]

(A) Biophysical properties of murine BALF in *Fasn*^{iΔAT2} and control *Sftp-c*^{CreERT2+/-} mice exposed to 6 weeks of smoke. Utilizing a sessile drop tensiometer, surface tension (IFT mN/m) of the fluid was calculated. (B) Alterations in percentage abundance of SFA, MUFA and PUFA containing PC species in BALF isolated from wild-type (WT), *Fasn*^{iΔAT2} and control *Sftp-c*^{CreERT2+/-} mice smoked or un-smoked for 6 weeks. Abundance was calculated by expressing the total concentration of each different fatty acids group as a percentage of total PC species. (C) Volcano plot analysis illustrating significantly altered PUFA phospholipids in BALF isolated from *Fasn*^{iΔAT2} and control *Sftp-c*^{CreERT2+/-} mice smoked for 6 weeks. Threshold: adjusted p-value < 0.05, log2fold change > 2. Data represented as mean ± SEM. ####P < 0.001, ###P < 0.01, #P < 0.05 and ns = non-significant, by one-way ANOVA with Tukey's correction. Each data point represents an individual mouse, n=4-5 mice per group.

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Lung tissue can also be used to assess AT2 cell function by examining AT2-specific proteins like Pro-SFTP-C. Loss of *Fasn* AT2 cells led to decreased Pro-SFTP-C expression in whole lung tissue (**Figure 3.12A**), suggesting potential disruptions in the synthesis of the hydrophobic SFTP-C protein, which may contribute to the previously observed increased surface tension in BALF.



Taken together these results underscore the advantage of using lung-specific SFTP-C promoter to modulate AT2 cell function in both health and disease models in mice. AT2 cells can be isolated for direct study, or their function can be indirectly assessed through BALF and lung tissue analysis.

3.3 Discussion

AT2 cells are specialized epithelial cells in the lung alveoli. Through their diverse functions and communication with resident cells, AT2 cells are central to alveolar health. Their importance is underscored in disease states, where loss or dysfunction of AT2 cells leads to significant pathogenic consequences (8), emphasizing the need for continued research into their biology and role in lung health and disease. Currently, the lack of standardized methodologies in the field hinders reproducibility and comparability.

With a focus on surfactant biology, we examined the utility of lipidomic analyses and AT2-specific gene-deletion systems to investigate AT2 function under both healthy and disease conditions. Utilizing BALF and lung tissue, we indirectly assessed AT2 function, uncovering how disruptions in lipid metabolism, such as those caused by *Fasn* deletion, exacerbate surfactant dysfunction and lung injury in CS-induced conditions. Additionally, we leveraged the murine MLE-12 cell line as a cost-effective *in vitro* model. Through this system, we established a range of surfactant-assays, demonstrating the ability of MLE-12 cells to secrete, uptake, and produce surfactant-associated lipids and proteins.

Additionally, we investigated AT2 cell repair and regeneration using a wound-healing assay in MLE-12 cells and an AT2-to-AT1 cell differentiation protocol in primary mouse AT2 cells. By combining *in vitro* and *in vivo* approaches, we developed a comprehensive and accessible “tool-kit” for studying AT2-specific functions. This chapter highlights the value of employing a multimodal approach when studying AT2 biology, integrating a range of readouts to draw robust conclusions.

3.3.1 Surfactant Readouts

Using MLE-12 cells, a murine AT2-like cell line, we explored their capacity for surfactant secretion and uptake. Previous studies demonstrated that MLE-12 cells secrete PC upon stimulation with surfactant secretagogues like PMA (244). Building on this, we confirmed increased PC secretion following PMA treatment using a commercially available PC assay kit. Complementary pendant drop tensiometric analyses showed that PMA-stimulated supernatants reduced surface tension, indicating the presence of surfactant-active components. Furthermore, PMA stimulation also increased the secretion of mSFTP-C. mSFTP-C is a highly hydrophobic 3.7 kDa protein critical to surfactant function. Synthesized as a 21 kDa pro-protein, it undergoes proteolytic processing through various cellular

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compartments, before its packaging within lamellar bodies for secretion (**Figure 3.2A**) (3). Functionally, mSFTP-C enhances the surface activity of surfactant lipids by facilitating their adsorption at the air-liquid interface (3, 255). Despite its critical role, studies frequently focus on the pro-form of SFTP-C using immunoblotting, which limits insights into the kinetics and regulation of SFTP-C secretion by AT2 cells. Our findings are the **first** to demonstrate its active secretion in MLE-12 cells in response to PMA.

Extensive studies in primary AT2 cells and *in vivo* models have established the alveolar epithelium as a critical site for surfactant reuptake and recycling (3, 32, 247, 256, 257). Historically, studies have utilized radiolabelled PC to assess the clearance and uptake of PC lipids both *in vitro* and *in vivo* systems (3, 256, 257). To investigate surfactant uptake in MLE-12 cells, we employed a fluorescent NBD-DPPC assay, demonstrating for the **first time** that MLE-12 cells actively internalize DPPC. This simple but effective assay provides a valuable framework for exploring surfactant lipid uptake under various experimental conditions.

Next utilizing lipidomic analysis to highlight its utility in studying alterations in the AT2 cell lipidome under pathological conditions. Lipidomic analyses of AT2 cells from healthy mice corroborated previous findings, identifying PCs and FC as the dominant lipid classes, with sphingolipids and glycerolipids as minor components (209, 248). This technique offers a broad overview of lipid species present, extending beyond individual molecules, and highlighting specific lipids that are contributing to pathological conditions. In AT2 cells isolated from CS-exposed mice, surfactant lipid abundance significantly declined, while ether phospholipids (etherPLs), triglycerides (TGs), and SM species, lipids previously linked to COPD pathology, showed marked increases[€].

Notably, PUFA-containing lipids were substantially elevated in CS-exposed cells, which are susceptible to lipid peroxidation[€]. This altered lipidome, characterized by increased PUFA-lipids, may contribute to ferroptosis, an iron-dependent lipid peroxidation-driven cell death pathway which has previously been associated with CS-induced AT2 cell injury (210). These subtle lipidomic shifts, which would be missed in conventional assays, underscore the value of lipidomics.

An additional powerful tool in AT2 cell biology is the use of the SFTP-C promoter in inducible Cre mouse models, enabling targeted gene deletion specifically in AT2 cells. This approach provides a platform to dissect gene-specific roles in AT2 cell and lung function. Previous studies demonstrate that AT2-specific gene deletions impair cellular function, injury

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responses, and the lung microenvironment, with effects on overall pulmonary health (69, 209, 258).

Using this system, we generated a model of disrupted surfactant synthesis by deleting *Fasn*, a key enzyme for *de novo* fatty acid synthesis, specifically in AT2 cells. As shown in Fan *et al.* (2024), integrating lipidomics, surface tension analysis, and BALF and lung tissue assessments revealed that a loss of *Fasn* in AT2 cells exacerbates surfactant imbalance and lung damage in smoke-exposed mice, highlighting a role for AT2 lipid metabolism in modulating the lung's response to cigarette smoke[€]. Beyond the discussed findings, *Fasn*^{*ΔAT2*} mice displayed significantly reduced ProSFTP-C protein levels in lung tissue. Lipidomic profiles of BALF from *Fasn*^{*ΔAT2*} mice also showed decreased levels of specific MUFA and PUFA lipids including acylcarnitines, diacylglycerols, ceramides, and phospholipid derivatives, suggesting disruptions in surfactant lipid homeostasis[€]. Therefore the reduction in ProSFTP-C may stem from disrupted mSFTP-C-surfactant lipid interactions due to the altered surfactant lipidome or increased protein turnover; further analysis of secreted mSFTP-C levels in BALF could clarify these mechanisms.

Biophysical analyses indicated increased surface tension in BALF from *Fasn*-deficient mice, a phenotype exacerbated by smoke exposure. We reported that this was due to lower DPPC levels in the BALF, resulting in increased surface tension levels in the BALF, damaging the epithelium[€]. In the pathophysiology of COPD, particularly in cases of emphysema, patients experience hyper-inflammation driven by the loss of elastic recoil in the lungs (259). This loss results in impaired alveolar function, reduced ability to expel air, and consequent air trapping, which exacerbates inflammation and contributes to disease progression. Here we suggest increased lipid species results in increased maximum surface tension capacity which could enhance alveolar expansion linking these changes to the hyperinflation phenotype observed in conditions such as emphysema.

Integrating lipidomics, surface tension analyses, and functional assays, this study highlights the critical interplay between lipid metabolism and surfactant functionality in AT2 cells. While MLE-12 cells offer a scalable and cost-effective model for initial studies, the detailed insights provided by primary AT2 cells and *in vivo* models remain indispensable for understanding the full complexity of AT2 cell biology. Together these findings underscore the need for multimodal approaches combining both *in vitro* and *in vivo* systems to address the challenges in studying AT2-specific functions and their implications in lung health and disease.

€ - Where indicated, portions of this text and data were previously published, to which I contributed primary analysis and writing (please see declaration page i). Fan, L.C., et al., Alveolar type II epithelial cell FASN maintains lipid homeostasis in experimental COPD. JCI 134(2023) 6140.

3.3.2 Repair Readouts

As discussed, the most robust methods for investigating AT2 cell stem-cell capacity currently involve complex lineage-tracing models or *ex vivo* alveolar organoid cultures; however, both approaches are technically complex and resource-intensive. As a simpler alternative, we employed an *in vitro* wound-healing assay using MLE-12 cells. Following wounding, MLE-12 cells migrated, proliferating to close the wound within 48 hours. Although the scratch assay is a simple technique, its flexibility allows adaptation to various cell types and experimental setups (228, 260).

A limitation of cell lines is their terminal differentiation state consisting of a heterogeneous population expressing both AT1 and AT2 markers, making it difficult to study AT2 cells capacity to transdifferentiate into AT1 cells. In contrast, the transdifferentiation capacity of primary AT2 cells can be effectively studied *ex vivo*, where isolated AT2 cells progressively lose their AT2 characteristics and transition into AT1 cells (238, 239). Consistent with existing literature, we observed a transient increase in the transitional marker *Krt8* at day 5, alongside a gradual rise in AT1 marker *Aqp5*, peaking after one week. This *ex vivo* model is advantageous as it can be applied to different complex mouse models or under varied conditions, such as altered micronutrient availability. However, a key limitation is the labour-intensive isolation process, requiring large cell numbers and pooling from multiple mice (typically 3–4).

3.4 Limitations

While MLE-12 cells offer a cost-effective platform to study surfactant biology, they cannot fully replicate primary AT2 cell functionality. For example, studies with A549 cells, often used as a human AT2-like model, have shown differences in phospholipid composition and surfactant activity compared to primary AT2 fractions (241). MLE-12 cells are more representative of AT2 cells compared to A549 cells (244, 261), as they have been shown to secrete surfactant lipids upon stimulation, which was confirmed in this study (**Figure 3.2E**). Furthermore, this study also highlighted the cells capacity to secrete mSFTP-C and uptake surfactant lipids (**Figure 3.2C, 3.2D and 3.3D**).

Surfactant secretion assays developed in MLE-12 cells should be repeated in other cell types to confirm whether the observed phenomena are unique to AT2-like cells or cells in culture. In PMA-induced secretion assays, despite its established role in stimulating surfactant secretion potential off-target effects of PMA must be considered (262, 263). Validation using alternative secretagogues could strengthen these findings (3, 223).

A key limitation of the NBD-DPPC assay is that the fluorescent probe is not a native lipid, and the attached fluorophore may alter its transport properties, as seen in other NBD-based nutrient uptake assays (264). Previous work in the Cloonan lab demonstrated that treatment with unlabelled DPPC reduced the uptake of fluorescent DPPC, indicating competition for the same uptake pathway as the native molecule. While this critical control has been performed for other cell types, it was not feasible to include here due to resource constraints. Recent advancements, such as the Click-IT chemistry approach, address this issue by enabling transport of minimally modified nutrients, followed by fluorescent labelling after cell uptake (224, 265). This technique offers additional advantages, including the ability to label precursors like the choline head group, which can track both uptake efficiency and incorporation into phospholipids (265). While further refinement is needed to make the Click-IT method more accessible, it holds significant promise for future studies.

Comprehensive analysis of surfactant remains challenging due to its complex composition of lipids, surfactant proteins, and other proteins (e.g., serum-derived). Lipidomics offers insights, but isolating extracellular and intracellular surfactant is difficult. While BALF is enriched for surfactant, it is diluted in saline, and intracellular surfactant packaged in lamellar bodies often requires rigorous centrifugation, which is rarely performed (3). As a result, lipid species, which

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are enriched in surfactant are also present in other cellular components throughout the cell and therefore will be included in the signal. In this study, we focused on cell-free, lipid-rich BALF, primary mouse AT2 isolates and supMLE-12; and acknowledge the limitations. Advances such as single-organelle lipidomics in live cells via micro-Raman spectroscopy could significantly enhance future analyses, however the issue still remains of accessibility to these complex and expensive techniques (266).

The interpretation of surface tension measurements using the pendant drop tensiometer primarily reflects the maximum surface tension of the fluid. For BALF, this is relevant because the fluid is adapted to line the alveoli, and maximum surface tension offers insight into its biophysical properties. However, the analysis of supMLE-12 media has limitations due to dilution of surfactant-like substances during exocytosis.

We concluded for supMLE-12 surface tension measurements based on findings from PC-assays and secreted mSFTP-C, which have previously been associated with reduced surface tension, that a lower surface tension is a result of the active secretion of surfactant into the medium.

3.5 Conclusion

This study advances our understanding of AT2 cell biology by evaluating methodologies that integrate *in vitro* and *in vivo* systems to address key questions in lung health and disease. By leveraging MLE-12 cells and primary AT2 cells, we have developed scalable models to study surfactant biology, lipid metabolism, and cellular repair mechanisms. Lipidomic analyses and functional assays revealed critical links between lipid metabolism and surfactant functionality, underscoring the importance of AT2 cell lipid homeostasis in maintaining lung integrity under stress, such as cigarette smoke exposure.

Biophysical assessments highlighted how disruptions, such as *Fasn* deletion in AT2 cells, exacerbate surfactant dysfunction, increase surface tension, and contribute to epithelial damage. Repair-focused assays demonstrated the utility of MLE-12 cells in migration studies, while primary AT2 cells enabled exploration of differentiation dynamics.

Together, this chapter developed an accessible **AT2-specific tool-kit** for *in vitro* and *in vivo* studies, establishing standardized protocols and pipelines to investigate AT2 cell functions. By addressing the strengths and limitations of current techniques, this chapter highlights the value of integrative, multimodal approaches to advance our understanding of AT2 cell biology.

4. Ironing Out the Role of Fe in AT2 Cell Function *in Vitro*.

4.1 Introduction

Iron is a fundamental element essential for numerous cellular processes, including oxygen transport, DNA synthesis and energy metabolism. Its dual role as a vital nutrient and a potential driver of oxidative damage necessitates tight regulatory mechanisms to ensure proper acquisition, transport, use and storage. While iron's importance in systemic and cellular functions is well-documented, relatively little is understood about its role in specialized lung cells like the AT2 cells (143). AT2 cells are essential for maintaining alveolar health, playing key roles in surfactant production and alveolar repair. The specialized functions of AT2 cells are highly energy-demanding, relying heavily on mitochondrial output. Notably, iron plays a central role in both cellular and mitochondrial metabolism. Despite this clear overlap, the connection between iron, particularly MITOIRON, and AT2-specific functions remains poorly understood, highlighting the need for further investigation.

AT2 cells, like most cell types, are thought to primarily acquire iron via transferrin-bound iron uptake through TFR1 (182). Additionally, the airway epithelium expresses other iron transporters, including DMT1 and ZIP14, along with the DCYTB, which likely facilitate the uptake of non-transferrin-bound iron (182, 190, 191). Surprisingly, as a by-product of investigations into AT2 cell surfactant recycling capacity, AT2 cells internalize ferritin (31). William (1984) demonstrated that neonatal rabbit AT2 cells accumulated both charged and native ferritin in lamellar bodies within 17 hours of instillation, underscoring their unique endocytic capacity. This suggests that AT2 cells may internalize by-products from the alveolar fluid, such as excess iron or iron-associated proteins, during surfactant turnover.

Once internalized, iron enters the labile iron pool, where it is either utilized for metabolic functions within organelles such as mitochondria or safely stored in ferritin nanocages composed of heavy and light chains (182). There is limited evidence regarding iron export from AT2 cells, with one study reporting no significant expression of FPN in the alveolar region (192). However systemic iron overload, induced by point mutations in ferroportin, results in iron accumulation in the alveolar region, thickening of the air-blood barrier, and AT2 cell hypertrophy and hyperplasia (188, 189); suggesting FPN/hepcidin regulatory axis contributes to iron handling in AT2 cells.

The role of iron in AT2 cell-mediated alveolar repair remains poorly understood. Recent findings by Zhuang *et al.* (2024) suggest that aging AT2 cells exhibit impaired differentiation into AT1 cells due to functional iron insufficiency (267). Using lineage-tracing models, they

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identified the transcriptional regulator NUPR1, an upstream mediator of LCN2, as a key suppressor of AT2 stemness (267). However, LCN2 is a secreted protein and is not exclusively indicative of iron deficiency, limiting interpretations of AT2 iron status in this context. Interestingly, an independent study identified a distinct subset of AT2 cells that upregulate Lcn2 expression following injury (75). These observations support a role for iron metabolism in alveolar repair, though the precise mechanisms remain unclear. It is plausible that LCN2 is secreted by AT2 cells either to mitigate iron-mediated oxidative damage during injury or to limit iron availability to tumour cells during age-associated tumorigenesis.

To date, there is no direct evidence linking iron metabolism to surfactant homeostasis in AT2 cells. However, characterization of the effects of surplus iron in the alveolar space highlighted that Fe^{3+} - transferrin alters the biophysical properties of BALF, increasing minimal surface tension capacity (268, 269). This excess iron in the alveolar space can disrupt surfactant lipids, through iron-catalyzed hydroxyl and lipid radical formation (268). In FPN-mutant mice, elevated iron levels were associated with reduced lamellar body volume in AT2 cells, though the total lamellar body-to-cell volume ratio remained unchanged, leaving unanswered questions about surfactant secretion, recycling, or composition (188). Additionally, models of BLM-induced fibrosis and cigarette smoke exposure show iron accumulation in AT2 cells and activation of ferroptosis, an iron-dependent, lipid peroxidation-driven cell death pathway (208-210). Given the lipid-rich nature of AT2 cells, these findings suggest they may be particularly vulnerable to iron-induced oxidative damage.

AT2 cells may also be susceptible to damage from heme-derived by-products. Cell free haemoglobin (CFH) increases alveolar permeability and inflammation disrupting barrier function *in vitro* and *in vivo* (186). In AT1 cells, excess heme-derived iron impairs mitochondrial metabolism and compromises barrier integrity *in vitro* (187), though whether similar effects occur in AT2 cells remains unknown. While several studies have linked iron overload to AT2 cell dysfunction, little is known about the role of iron in supporting their specialized functions. Given the high metabolic demands of AT2 cells and their reliance on mitochondrial function, it is likely that a significant proportion of intracellular iron is allocated to support mitochondrial metabolism and energy production. Leading to the question; **Is there consequences of iron deficiency - specifically MITOIRON deficiency - in AT2 cell biology?**

4.1.2 Chapter Aims and Objectives

An overarching theme in the literature is the minimal investigations into iron metabolism in the alveolar epithelium. While recent studies have linked iron dysregulation to AT2 cell dysfunction, often characterized by senescent markers, they fall short of comprehensively addressing how these disruptions impact AT2 specific functions, such as surfactant homeostasis and alveolar repair. A key limitation in many of these studies is their lack of targeted approaches to specifically manipulate iron levels in AT2 cells, relying instead on global inhibitors that obscure the precise cellular or organ-specific effects of iron dysregulation. Whether or not AT2 cells require iron to carry out their specialised function remains unknown.

To address this gap, we investigated the role of iron and MITOIRON in AT2-specific processes described in **Chapter 3**. Using MLE-12 cells as an *in vitro* AT2 model, we manipulated intracellular iron levels through ferric ammonium citrate (FAC) supplementation and iron chelation with deferoxamine (DFX) or deferiprone (DFP). Additionally, CRISPR-Cas9 knockdown of mitochondrial iron transporters (Mitoferrin 1/2) was employed to disrupt mitochondrial iron metabolism. By applying the AT2-specific toolkit developed in **Chapter 3** to these conditions, this chapter aims to elucidate the role for iron in AT2 cell surfactant homeostasis and repair capacity.

This chapter aims to;

1. Investigate how altered intracellular iron levels affect AT2 cell biology.
2. Explore the mechanisms of iron uptake and utilization in AT2 cells.
3. Establish an *in vitro* model of disrupted MITOIRON and examine how it alters AT2 cell specific functions.

4.2 Results

4.2.1 Investigating How Increased Intracellular Iron Levels Affect AT2 Cell Function *in Vitro*

There is limited literature on the role of iron in AT2 cell function. To address this, the impact of increased intracellular iron levels on AT2 cell function using MLE-12 cells as an *in vitro* model was examined.

4.2.1.1 Ferric Ammonium Citrate increases intracellular iron levels in MLE-12 cells

FAC was selected as an Fe^{3+} source. FAC treatment at increasing concentrations had minimal impact on MLE-12 cell viability after 24 hours (**Figure 4.1A**). To avoid excessive iron loading, we proceeded with 100 μM FAC for 24 hours in further experiments, a standard dose used

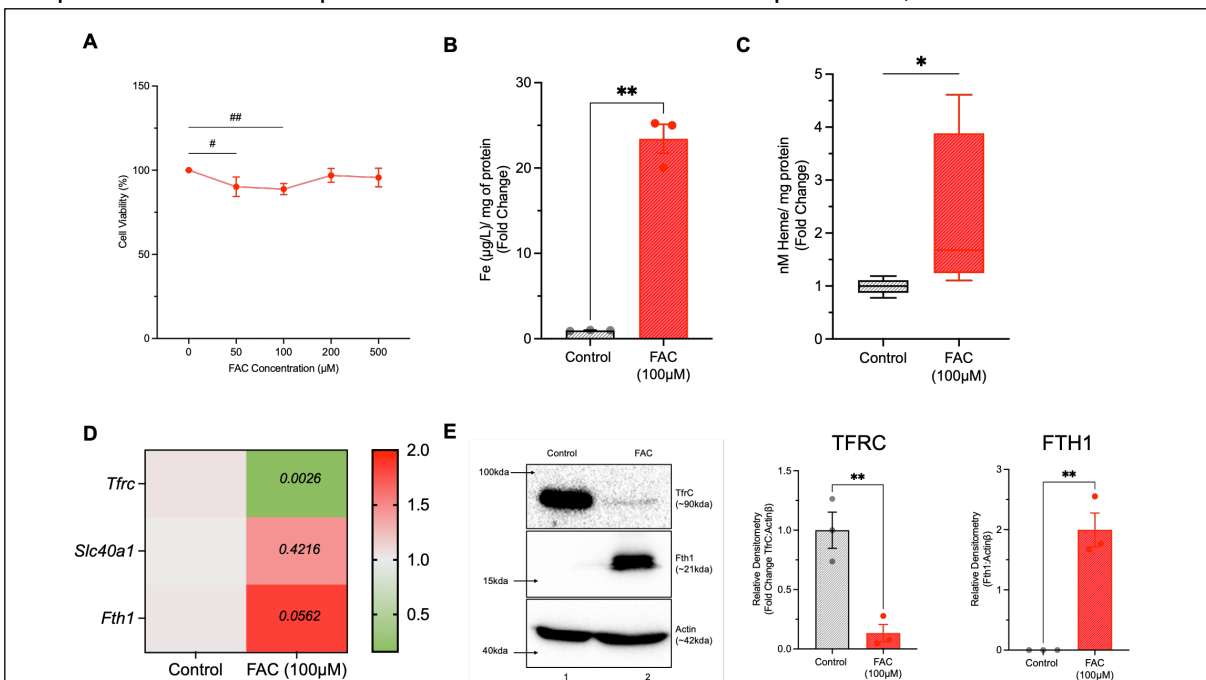


Figure 4.1: Ferric Ammonium Citrate increases intracellular iron and heme levels in MLE-12 cells

(A) Alamar Blue assay depicting cellular viability in MLE-12 cells when treated with increasing concentrations of FAC for 24hrs (B) GF-AAS analysis depicts total intracellular iron levels in MLE-12 cells after 24hr treatment with 100 μM FAC. (C) Total heme levels in MLE-12 cells after 24hr treatment with 100 μM FAC. Data represented as box plot min-to-max. (D) RT-qPCR data of iron genes *Tfrc*, *Slc40a1* and *Fth1* represented as foldchange (E) Representative immunoblot of TFRC and FTH1 expression in MLE-12 cells treated with FAC (100 μM) for 24hrs (left). Corresponding densitometry of Tfrc (left) and Fth1 (right) with β -Actin serving as a loading control (right). All data presented as mean $\pm$ SEM. P-value calculated by unpaired student t-test (*), or one-way ANOVA (#). ****P < 0.0001, ***P < 0.001, ###/*** P < 0.01, #/P < 0.05, ns = non-significant. Each data point corresponds to an individual biological replicate, with panel A, C and D n = 4 independent biological replicates.

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within the field (270, 271). 100 μ M FAC for 24 hours, equivalent to 0.0265 μ g/mL of iron. In our *in vivo* study, control mice had approximately 0.02053 μ g/mL of iron in their alveolar space (**Chapter 5, Figure 5.13**), consistent with reported literature values (182). While 100 μ M FAC is slightly higher than physiological levels, given that iron is distributed among alveolar cells, this treatment represents controlled iron-loading designed to mimic conditions of iron repletion without inducing excessive toxicity. Further analysis confirmed that this treatment significantly increased total intracellular iron (**Figure 4.1B**) and heme levels (**Figure 4.1C**). Additionally, the downregulation of iron uptake (*TfrC*) and upregulation of iron export (*Slc40a1*) and storage (*Fth1*) markers at both the protein and transcript levels highlighted an iron-loaded phenotype (**Figures 4.1D and 4.1E**).

4.2.1.2 Iron loaded MLE-12 cells have a reduced surfactant uptake and wound healing capacity

After establishing an iron-loading *in vitro* model, we applied the assays developed in **Chapter 3** to assess how increased intracellular iron affects MLE-12 cell function. Elevated iron levels

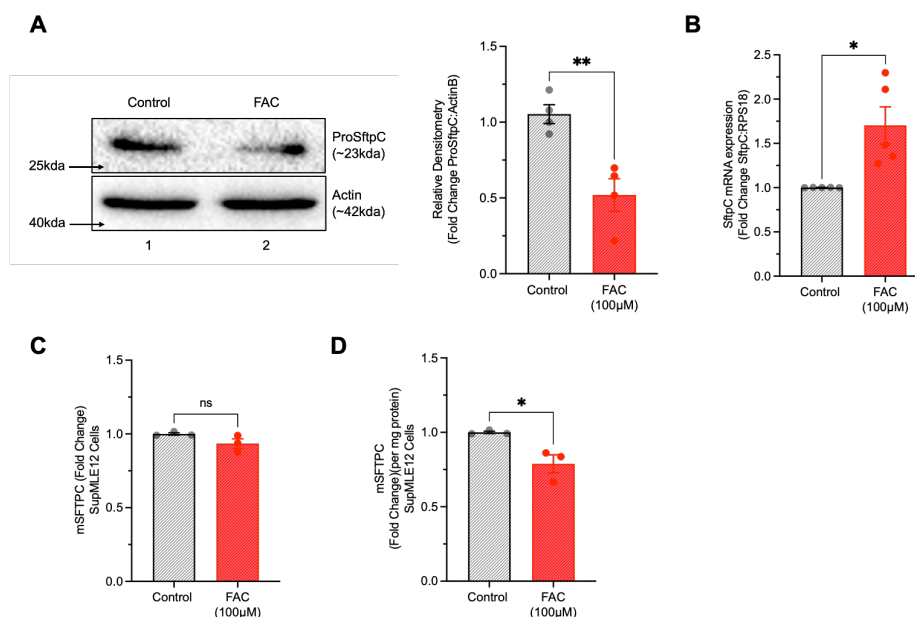


Figure 4.2: Increased intracellular iron levels in MLE-12 cells reduces Pro-SFTPC and mSFTPC secretion

(A) Representative immunoblot (left) and relative densitometric quantification (right) for AT2 cell functional marker Pro-SFTPC with housekeeping control β -actin in MLE-12 cells after FAC treatment. (B) Gene expression of *Sftpc* in MLE-12 cells after 100 μ M FAC treatment measured by RTqPCR. (C) Secreted *Sftpc* in supernatant of MLE-12 measure by ELISA. Cells pre-treated with FAC(24hrs) washed and supplemented with new media overnight. (D) mSFTPC levels in supMLE-12 normalised to protein levels to account for cells lost in treatment group. All data presented as mean $\pm$ SEM. P-value calculated by unpaired student t-test. **P < 0.01, *P < 0.05, ns = non-significant. Each data point represents an individual biological replicate. Abbreviations: Mature Sftpc (mSFTPC), Supernatant from MLE-12 cells (supMLE-12).

decreased pro-SFTPC protein expression (**Figure 4.2A**), increased *Sftpc* transcript levels

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(Figure 4.2B), but did not alter secreted protein levels (Figure 4.2C). Further analysis revealed a significant reduction in mSFTPC levels in supMLE-12 when normalized to total protein (Figure 4.2D), indicating decreased protein synthesis and secretion in iron replete conditions.

Transcript levels of surfactant/lipid genes trended towards increased uptake (*Cd36*) and synthesis of fatty acids (*Fasn*, *Fgfr2*) (Figure 4.3A), suggesting increased lipid synthesis. NBD-DPPC uptake in iron-replete MLE-12 cells was significantly decreased (Figure 4.3B). Surface tension analysis showed lower baseline levels in supMLE-12 (Figure 4.3C), with no change after exocytosis stimulation (Figure 4.3D), indicating iron-loading in MLE-12 cells may have increased secretion of surface-lowering molecules, such as surfactant, at baseline.

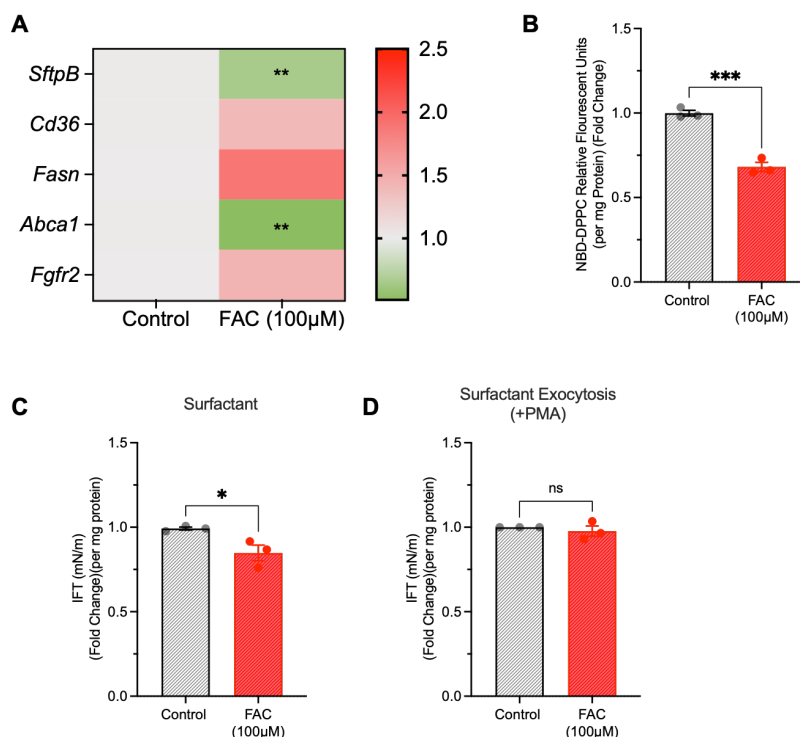


Figure 4.3: FAC perturbs surfactant uptake in MLE-12 cells

(A) RTqPCR data of surfactant and AT2 related genes; *Sftp-b*, *Cd36*, *Fasn*, *Abca1* and *Fgfr2* represented as foldchange, (n=4 biological replicates). (B) NBD-DPPC uptake in MLE-12 cells after FAC treatment. (C) Surface tension of iron-loaded MLE-12 cells was assessed after incubation with 100 μM FAC for 24hrs, followed by washing and overnight recovery in fresh medium. For stimulation of surfactant exocytosis, cells pre-loaded with 100 μM FAC for 24hrs, washed and supplemented with fresh medium. Cells were then treated with 10nM of PMA for 2hrs and the supernatant collected for surface tension readouts. For C and D surface tension levels were represented as mean of fold-change normalised to mg of protein, to account for cells lost during treatment. All data represented as mean ± SEM. P-value calculated by unpaired student t-test, ***P < 0.0001, **P < 0.01, *P < 0.05, ns = non-significant. Each data point represents and individual biological replicate for Figure 4.3B – 4.3C.

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To investigate the impact of iron loading on the regenerative capacity of MLE-12 cells, we utilized a wound-healing assay described in **Chapter 3**. MLE-12 cells were either pre-treated with 100 μ M FAC for 24 hours before injury, followed by HITES medium with 100 μ M FAC, or supplemented post-injury with the same medium. Iron-loading in MLE-12 cells significantly impaired wound healing capacity (**Figure 4.4A – 4.4C**). Post-injury FAC-treated cells had delayed wound closure but fully recovered by 48 hours. Moreover, pre-iron loaded cells also exhibited reduced early proliferation, in contrast these cells failed to fully close the wound after 48hrs (**Figure 4.4C**). Taken together these results suggest that iron loading impairs both surfactant turnover and the proliferative/repair capacity of MLE-12 cells.

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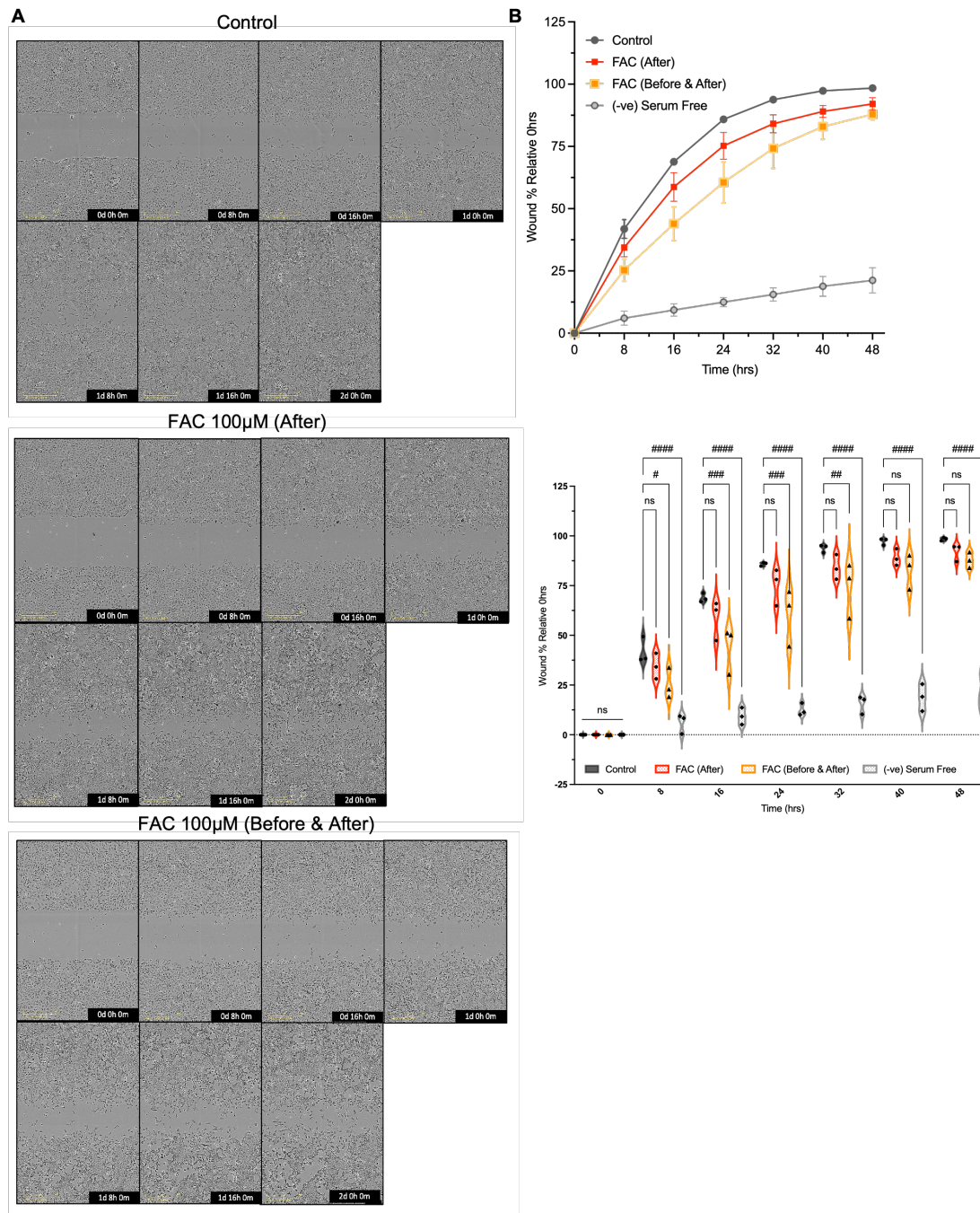
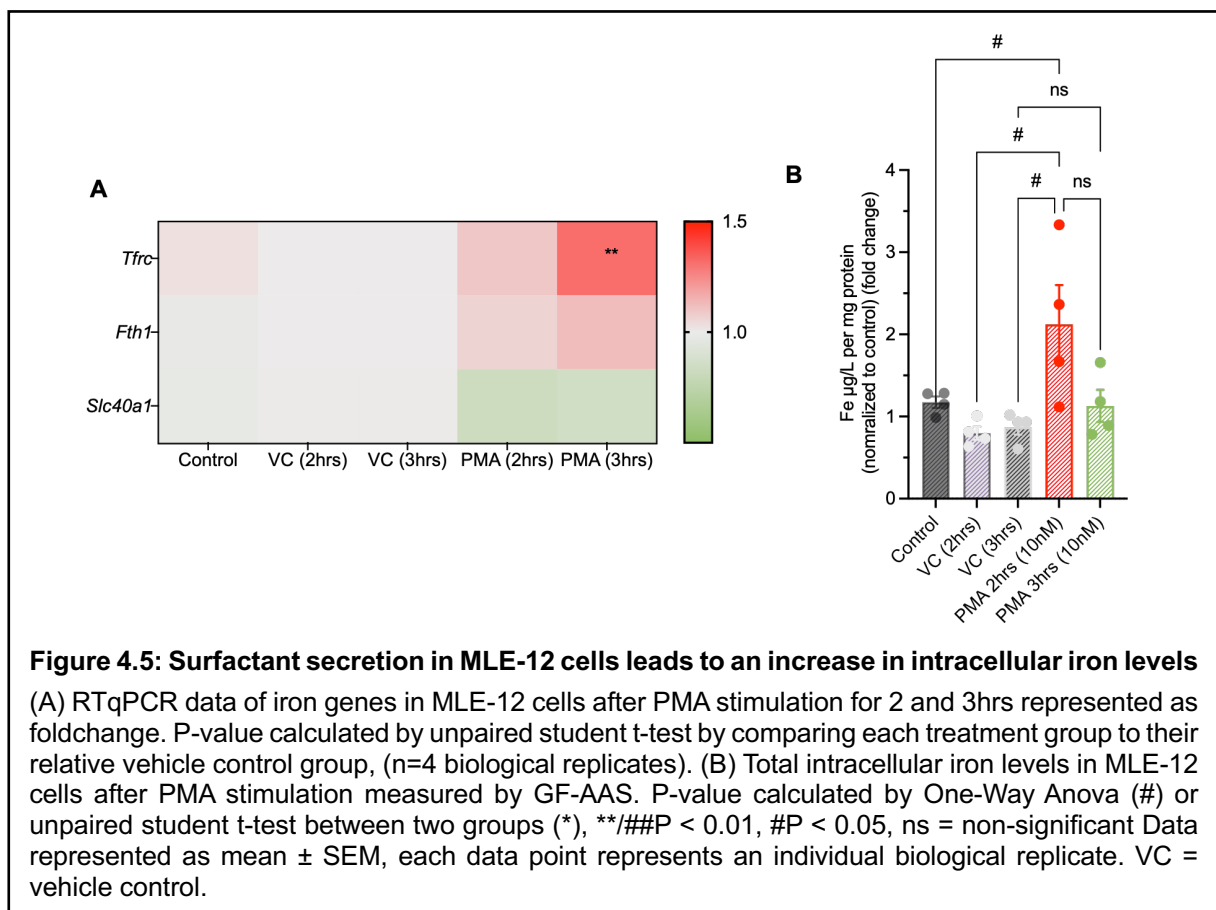


Figure 4.4: Iron loading in MLE-12 cells decreases their wound healing capacity

(A) Representative images of MLE-12 cells subjected to a scratch performed by the Incucyte® 96-Well Woundmaker Tool. Images were taken every 8hrs for 48hrs. Block of images include control cells (top), cells treated with 100µM FAC directly after scratch (middle), cells treated with 100µM FAC both 24hrs before and directly after scratch (bottom). Images captured at 10X. (B) Graphical representation illustrating the kinetic movement of cells and wound closure over time utilising the Satrious Essenbio software. Initially, the software generates a wound mask to identify the wound's edge. Subsequently, an algorithm calculates wound confluence (%), representing the fractional area of the wound occupied by cells at specific time points. Data represented as mean $\pm$ SEM, $n = 3$ independent biological replicates. (C) Grouped analysis to evaluate statistical significance between the treatment groups at the given times. Data represented as violin plot, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, $\#\#\#\#P < 0.0001$ ns = non-significant, by two-way ANOVA with Tukey's correction. All data represented as mean $\pm$ SEM.

4.2.2 Intracellular Iron Levels Are Increased During Surfactant Secretion in MLE-12 Cells.

Following the observation that increased intracellular iron levels in MLE-12 cells reduced surface tension in supMLE-12, we aimed to investigate how iron uptake and utilization might influence surfactant secretion in these cells. To stimulate surfactant release, MLE-12 cells were treated with the surfactant secretagogue PMA, and iron uptake and utilization was assessed at 2 and 3 hours post-stimulation. Transcriptional analysis showed a trend towards a time-dependent increase in iron uptake (*TfrC*) and storage (*Fth1*), along with decreased iron export (*Slc40a1*) (**Figure 4.5A**). Evaluation of intracellular iron levels by GF-AAS showed significantly increased iron after 2 hours, with levels returning to baseline by the 3-hour mark (**Figure 4.5B**). This data suggests that MLE-12 cells may require an increase in iron uptake and utilization during surfactant exocytosis at 2hrs.



4.2.3 Investigating How Decreased Intracellular Iron Levels Affects AT2 Cell Function *in Vitro*

After observing the effects that increased iron levels had on surfactant maintenance and regenerative capacity in MLE-12 cells, we sought to determine if iron depletion would have the opposite effect.

4.2.3.1 Iron chelators effectively reduced intracellular iron levels in MLE-12 cells.

Using two iron chelators, DFX for extracellular iron and DFP for intracellular iron (272). We found that cell viability decreased in a dose-dependent manner, with DFP showing a stronger impact at 100 and 200 μM test concentrations (**Figure 4.6A**). To avoid cell death influencing our results, 10 μM of DFP or DFX was used for functional studies. GF-AAS confirmed that both chelators significantly reduced intracellular iron levels (**Figure 4.6B**), while heme levels

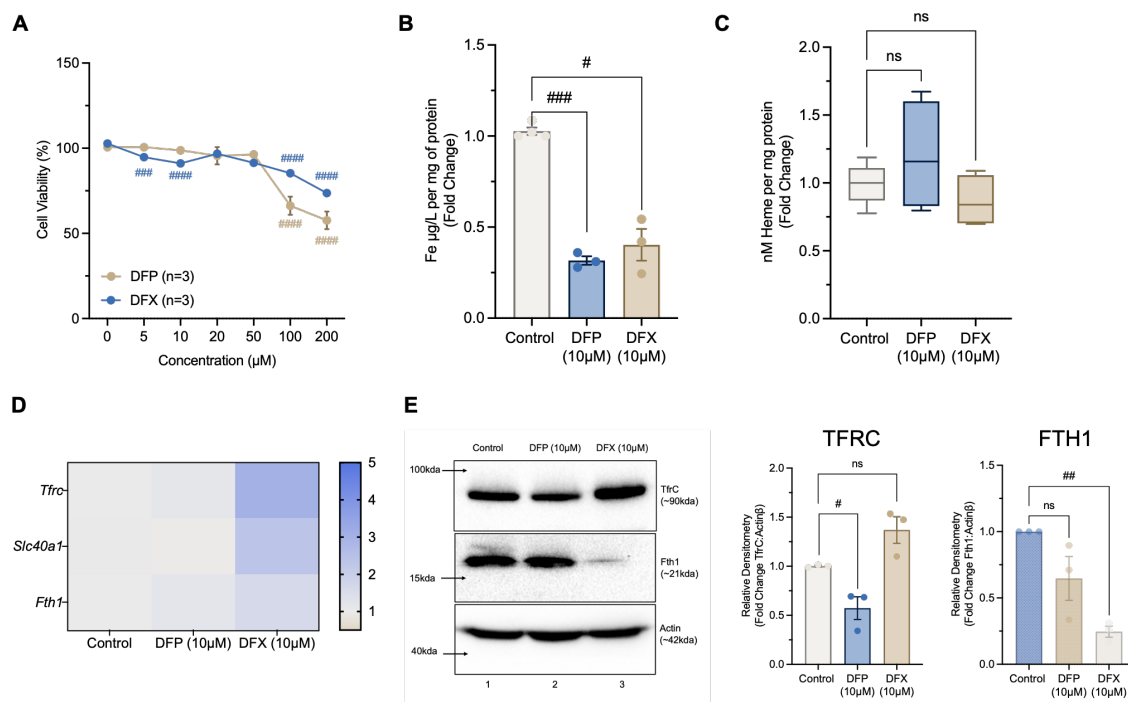


Figure 4.6: Intracellular iron levels are decreased in MLE-12 cells with both extracellular (DFX) and intracellular (DFP) iron chelation

(A) Cellular viability in MLE-12 cells when treated with increasing concentrations of DFP or DFX for 24hrs. (n=3 independent biological replicates). (B) GF-AAS analysis depicts total intracellular iron levels in MLE-12 cells after 24hr treatment with 10 μM DFP or DFX. (C) Total heme levels in MLE-12 cells after 24hr treatment with 10 μM DFP or DFX. Data represented as box plot min-to-max, n= 3 independent biological replicates. (D) RTqPCR data of iron genes *Tfr*, *Slc40a1* and *Fth1* represented as foldchange, n=4 independent biological replicates. (E) Representative immunoblot of TFRC and FTH1 expression in MLE-12 cells treated with 10 μM DFP or DFX for 24hrs (left). Corresponding densitometry for both TFRC and FTH1 with β -ACTIN serving as a loading control (right). All data presented as mean $\pm$ SEM. P-value calculated by P-value calculated by ordinary one-way ANOVA dunnett multiple comparisons test, ##### P < 0.0001, ### P < 0.001, ## P < 0.01, # P < 0.05, ns = non-significant.

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remained unchanged for both (**Figure 4.6C**). Interestingly, extracellular iron chelation, with DFX, altered iron metabolism by significantly increasing iron uptake machinery (*TfrC*) and downregulating iron storage pathways (*Fth1*), whereas DFP chelation showed no effect (**Figure 4.6D and 4.6E**). This was also observed at the transcriptional level (**Figure 4.6E**). Suggesting that intracellular iron chelation by DFP may not trigger cellular sensing of iron deficiency in MLE-12 cells.

4.2.3.2 Extracellular iron chelation perturbs surfactant exocytosis and wound healing capacity in MLE-12 cells

Iron depletion in MLE-12 cells had differential effects based on chelation type. Intracellular chelation trended towards increased pro-SFTPC protein levels (**Figure 4.7A**) without affecting

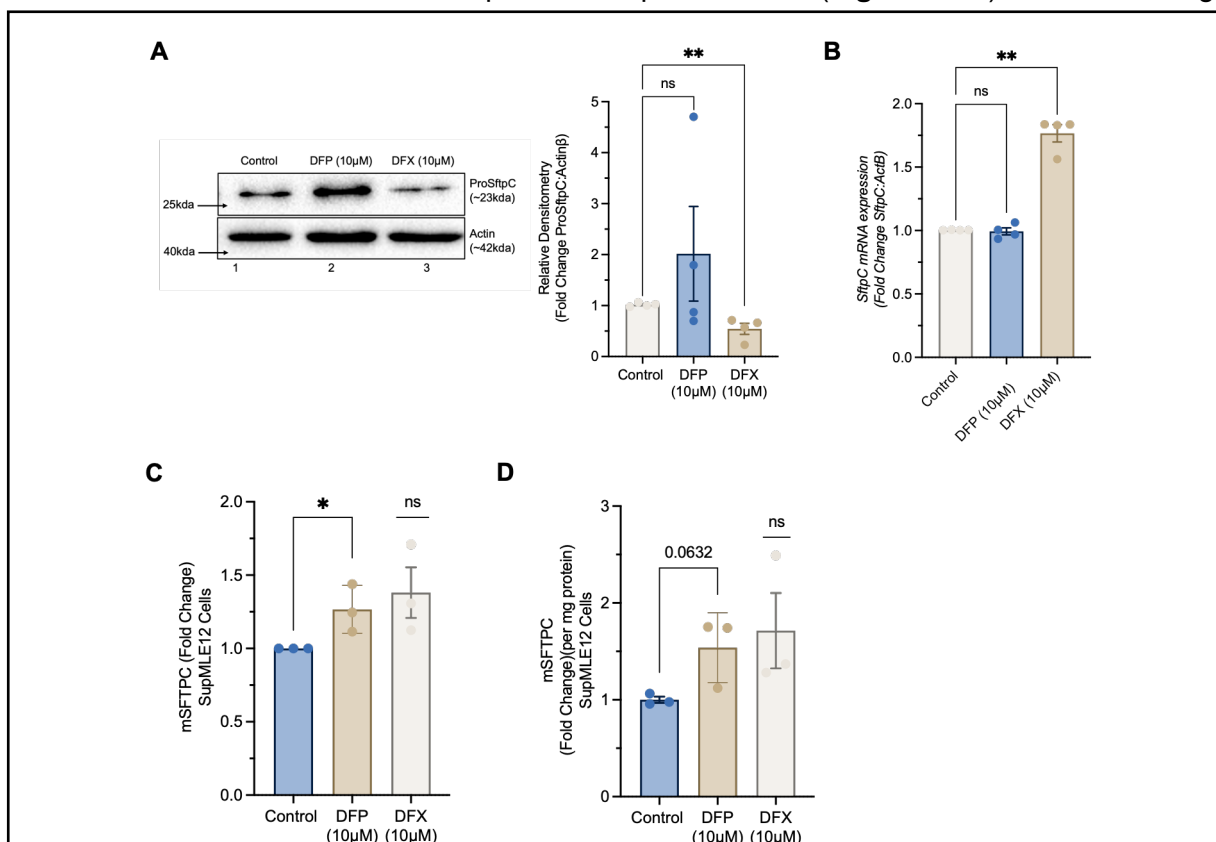


Figure 4.7: Extracellular iron chelation reduces Pro-SFTPC protein in MLE-12 cells

(A) Representative immunoblot (left) and relative densitometric quantification (right) for AT2 cells functional marker Pro-SFTPC with housekeeping control β -actin in MLE-12 cells. (B) Gene expression of *Sftpc* in MLE-12 cells after treatment with DFP or DFX for 24hrs. (C) Secreted Sftpc levels in levels in iron deplete supMLE-12. MLE-12 cells were treated with DFP or DFX for 24hrs. Cells were washed and supplemented with new media overnight, supernatant was harvested and secreted Sftpc levels quantified. (D) mSFTPC data represented as mean fold-change normalised to protein levels to account for changes in viability after treatments. P-value calculated by unpaired student t-test. * $P < 0.05$, ** $P < 0.01$, ns = non-significant. Each data point represents an independent biological replicate.

transcript levels (**Figure 4.7B**) but enhanced secretion of mSFTPC (**Figure 4.7C**), consistent even after normalization to total protein (**Figure 4.7D**). In contrast, extracellular chelation

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significantly reduced pro-SFTPC (**Figure 4.7A**), increased transcript levels (**Figure 4.7B**) and showed a trend toward increased secretion (**Figure 4.7C and 4.7D**).

Investigations into other surfactant genes showed that both DFP and DFX upregulated *Fasn*, *Cd36* and *SftpB* expression, with DFX having a stronger effect (**Figure 4.8A**). Suggesting iron chelation might stimulate the cells to make more FA and take up more lipids to provide the building blocks for surfactant. However, the NBD-DPPC uptake assay was unchanged in both models (**Figure 4.8B**). Surface tension was unchanged at baseline, however, PMA stimulation increased surface tension in iron depleted cells (**Figure 4.8C and 4.8D**). Indicating that iron chelation may impair surfactant exocytosis in MLE-12 cells.

Similar to iron-loading wound-healing assays, MLE-12 cells were divided into three groups for each chelator: (1) control, (2) pre-treated with 10 μ M DFP or DFX for 24 hours prior to injury

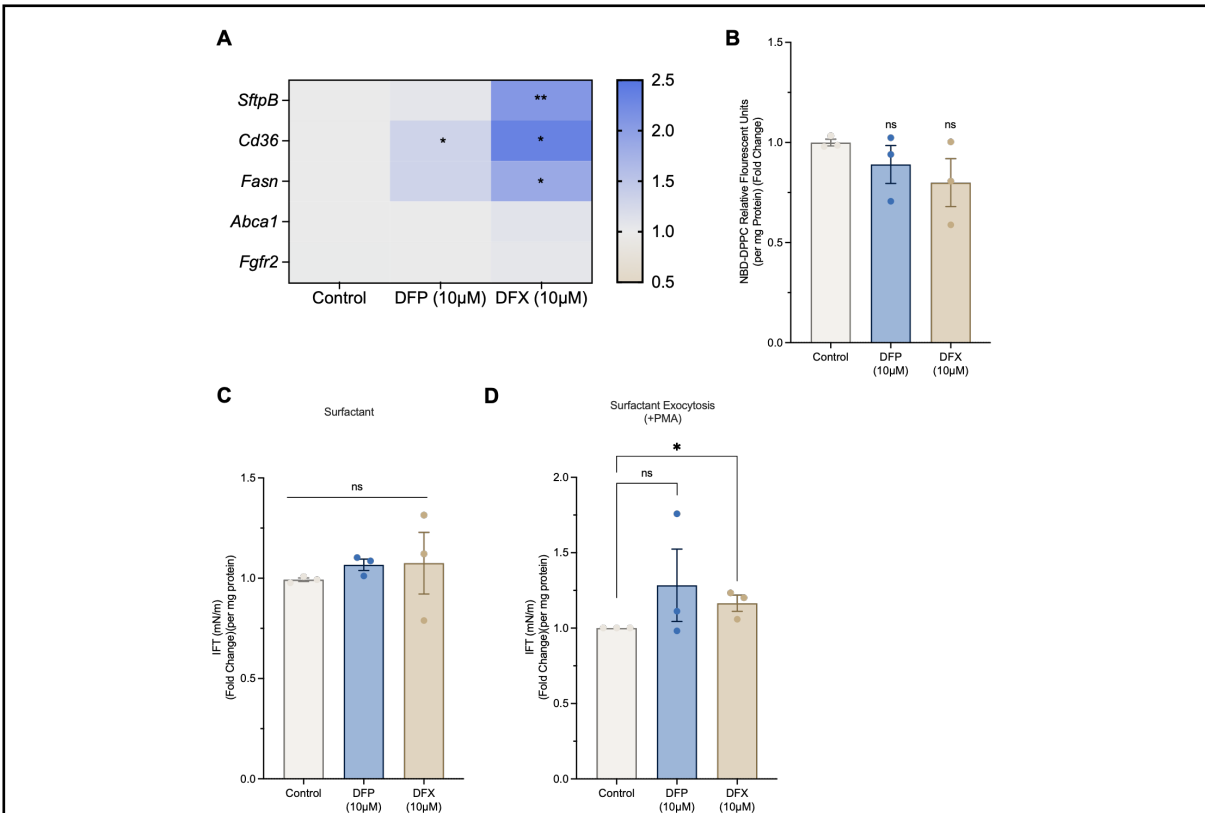


Figure 4.8: Extracellular (DFX) iron chelation stunts surfactant exocytosis in MLE-12 cells

(A) RTqPCR data of surfactant related genes; *Sftp-b*, *Cd36*, *Fasn*, *Abca1* and *Fgfr2* represented as foldchange, n=4 Biological replicates. (B) NBD-DPPC uptake in MLE-12 cells after DFP or DFX treatment. (C) Surface tension measurements of iron-depleted MLE-12 cells by treating the cells with 10 μ M of DFP or DFX for 24 hrs, followed by washing and overnight recovery in fresh medium. (D) To stimulate surfactant exocytosis, DFP- or DFX-treated cells were washed, replenished with fresh medium, exposed to 10 nM of PMA for 2hrs before collecting the supernatant for analysis. Data represented as mean of fold-change normalised to mg of protein, to account for loss of cells due to treatment. Fold change data represented as mean $\pm$ SEM P-value calculated by unpaired student t-test. *P<0.05, **P<0.01, ns = non-significant. Each data point represents an independent biological replicate.

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and continued post-injury with HITES medium containing the same chelator, and (3) treated only post-injury with the chelator-supplemented HITES medium. Notably, opposite effects were observed depending on the type of iron chelation. Intracellular iron chelation with DFP enhanced MLE-12 cell proliferation, accelerating wound closure, with full recovery achieved by 1 day and 16 hours compared to control cells (**Figure 4.9A - 4.9D**). In contrast, extracellular iron chelation with DFX impaired wound healing, significantly reducing closure at 24 hours when applied before or after injury, and preventing full recovery by 48 hours (**Figure 4.10A – 4.10D**).

These results underscore the unique effects different forms of iron depletion can have on MLE-12 cell function. Extracellular iron chelation impaired surfactant exocytosis and reduced wound healing capacity. Intracellular chelation similarly disrupted surfactant exocytosis but, unexpectedly, DFP enhanced wound healing, possibly due to off-target effects, warranting further investigation.

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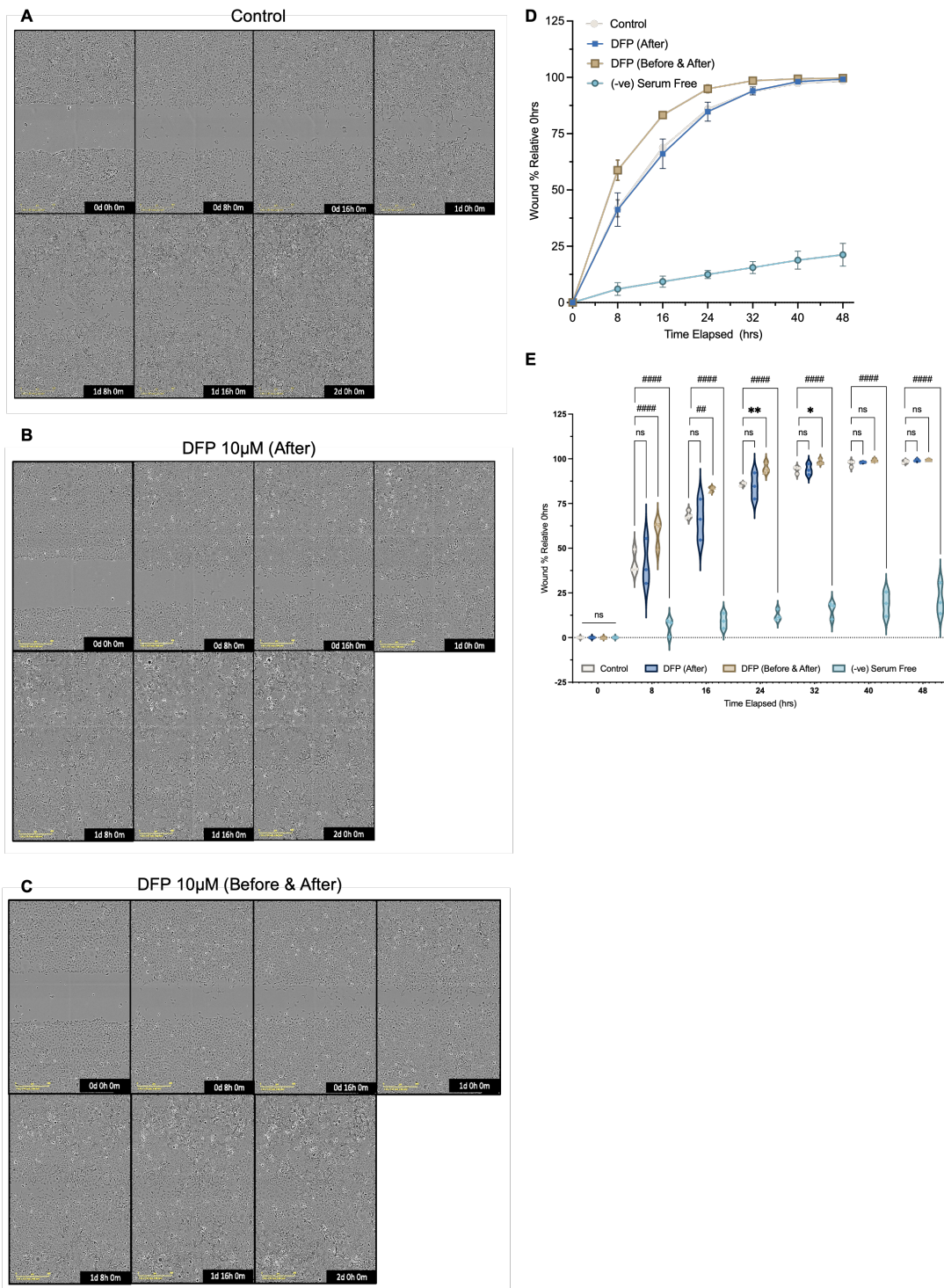
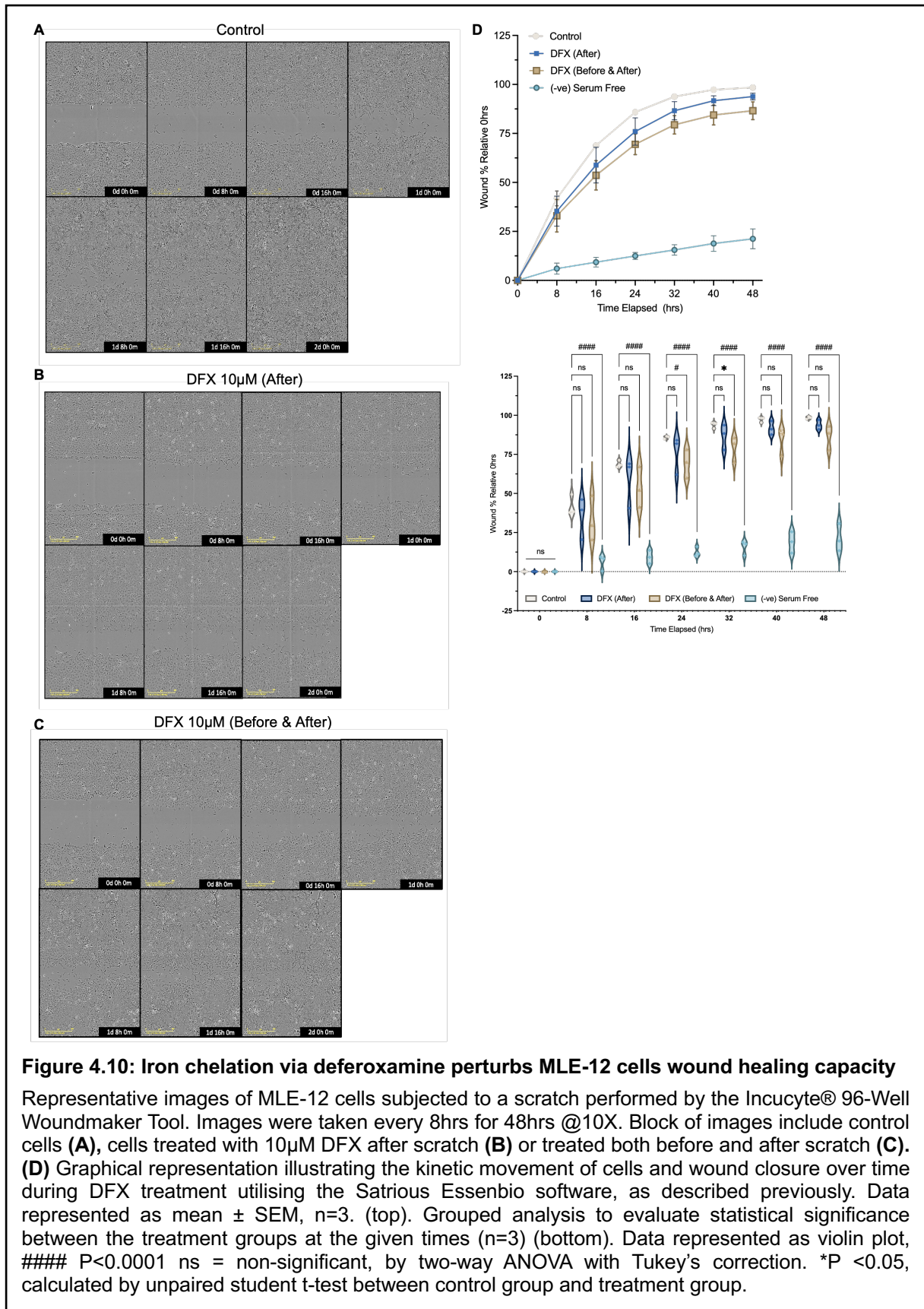


Figure 4.9: Deferiprone enhances MLE-12 cell wound healing capacity

Representative images of MLE-12 cells subjected to a scratch performed by the Incucyte® 96-Well Woundmaker Tool. Images were taken even 8hrs for 48hrs at 10X. Block of images include control cells (A), cells treated with 10µM DFP after scratch (B) or treated with DFP both before and after scratch (C). (D) Graphical representation illustrating the kinetic movement of cells and wound closure over time during DFP treatment utilising the Satrious Essenbio software, as described previously. Data represented as mean $\pm$ SEM, $n=3$. (E) Grouped analysis to evaluate statistical significance between the treatment groups at the given times ($n=3$). Data represented as violin plot, ####P < 0.001, ##### P<0.0001 ns = non-significant, by two-way ANOVA with Tukey's correction.

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4.2.4 Establishing an *in Vitro* Model of Dysregulated Iron Metabolism in MLE-12 Cells

Given the metabolic nature of AT2 cells and the established role of mitochondrial homeostasis in their function, we aimed to model mitochondrial iron perturbation. Iron import into mitochondria is mediated by the outer membrane transporters MFRN1 and MFRN2. Loss of either transporter has been shown to disrupt mitochondrial iron levels, impair heme biosynthesis, and alter cellular iron metabolism (149-152, 156). Therefore, utilizing CRISPRi technology we successfully silenced mitochondrial iron transporters, *Mfrn1* (*Slc25a37*) or *Mfrn2* (*Slc25a28*) (Figure 4.11A) as a model of MITOIRON dysregulation in AT2 cells.

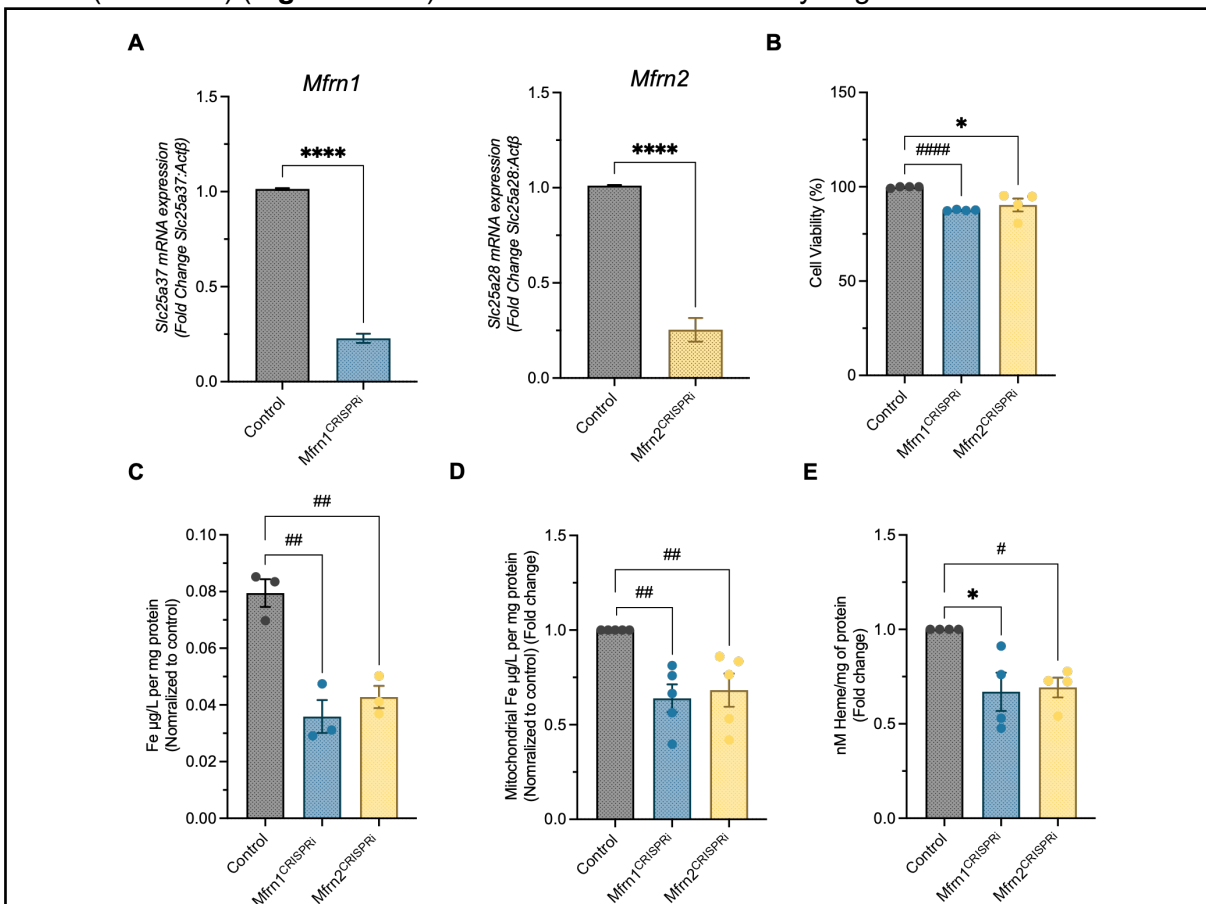


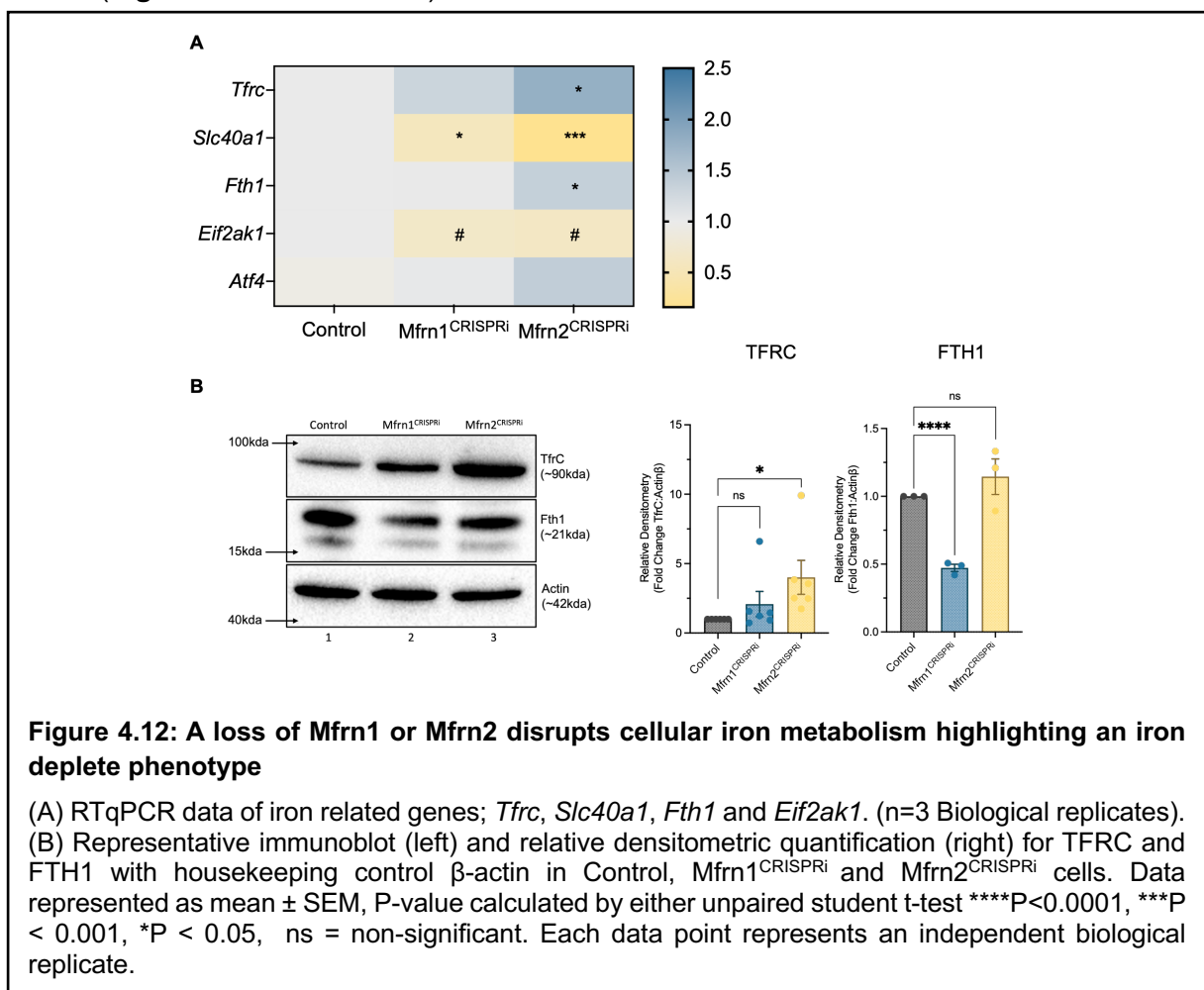
Figure 4.11: A loss of *Mfrn1* or *Mfrn2* in MLE-12 cells results in a decline in cell viability, decreased intracellular iron, mitochondrial iron and heme levels

(A) RT-qPCR qPCR analysis illustrates the stable knock down of mitochondrial iron transporters, *Mfrn1* (left) and *Mfrn2* (right), (n=6 independent biological replicates). (B) Alamar Blue assay details reduced cellular viability in both *Mfrn1*CRISPRi and *Mfrn2*CRISPRi cell lines when compared to controls. (C) Intracellular iron and (D) mitochondrial iron levels in both *Mfrn1*CRISPRi and *Mfrn2*CRISPRi cell lines, measured by GF-AAS. Data normalised to mg of protein. (E) Total heme levels represented as fold-change normalised to mg of protein in control, *Mfrn1*CRISPRi and *Mfrn2*CRISPRi cell lines. Data represented as mean $\pm$ SEM, P-value calculated by either unpaired student t-test (*) or one-way Anova (#) by Dunnett's multiple comparisons test. #####/****P<0.0001, ###/***P < 0.001, ##/**P < 0.01, #/*P < 0.05, ns = non-significant. Each data point represents an independent biological replicate.

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Depletion of either *Mfrn1* or *Mfrn2* led to reduced cellular viability (**Figure 4.11B**). GF-AAS analysis of whole-cell lysates and mitoplast isolates, i.e. mitochondria minus the outer mitochondrial membrane (214), revealed that loss of mitochondrial iron transport decreased both mitochondrial and cellular iron levels (**Figure 4.11C and 4.11D**). Intracellular heme levels were also significantly reduced in *Mfrn1*^{CRISPRi} and *Mfrn2*^{CRISPRi} cell lines when compared to control cells (**Figure 4.11E**).

Further investigation into iron metabolism regulators depicted an iron-deficient phenotype in *Mfrn1*^{CRISPRi} cells. A significant decrease in iron exporter (*Slc40a1*) mRNA was observed no significant changes in iron uptake (*TfrC*) or storage (*Fth1*) (**Figure 4.12A**). Whilst no significant change in TFRC iron uptake protein was observed, a significant reduction in FTH1 protein was noted (**Figure 4.12B**). Surprisingly, *Eif2ak1/Hri*, which is responsive to decreased intracellular heme and iron, was significantly decreased in both CRISPRi cells when compared to controls. *Mfrn2*^{CRISPRi} cells showed more pronounced changes in iron regulators compared to controls, except for unchanged FTH1 protein levels, possibly indicating impaired iron release from ferritin (**Figure 4.12A and 4.12B**).



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4.2.4.1 A loss of mitochondria iron transport disrupts surfactant maintenance; including surfactant secretion, uptake and production.

After genetically establishing an *in vitro* model of dysregulated iron metabolism, we investigated how the loss of mitochondrial iron transport, which decreases intracellular iron and heme levels, impacts MLE-12 cell surfactant homeostasis. Analysis of the key AT2 protein SFTPC revealed that loss of *Mfrn1* or *Mfrn2* disrupted the synthesis of its pro-form (**Figure 4.13A**). This was accompanied by a trend of *Sftpc* transcriptional upregulation, possibly as a compensatory response particularly in the *Mfrn2*^{CRISPRi} cell line (**Figure 4.13B**). However, baseline levels of the mature protein secretion remained unchanged (**Figure 4.13C**).

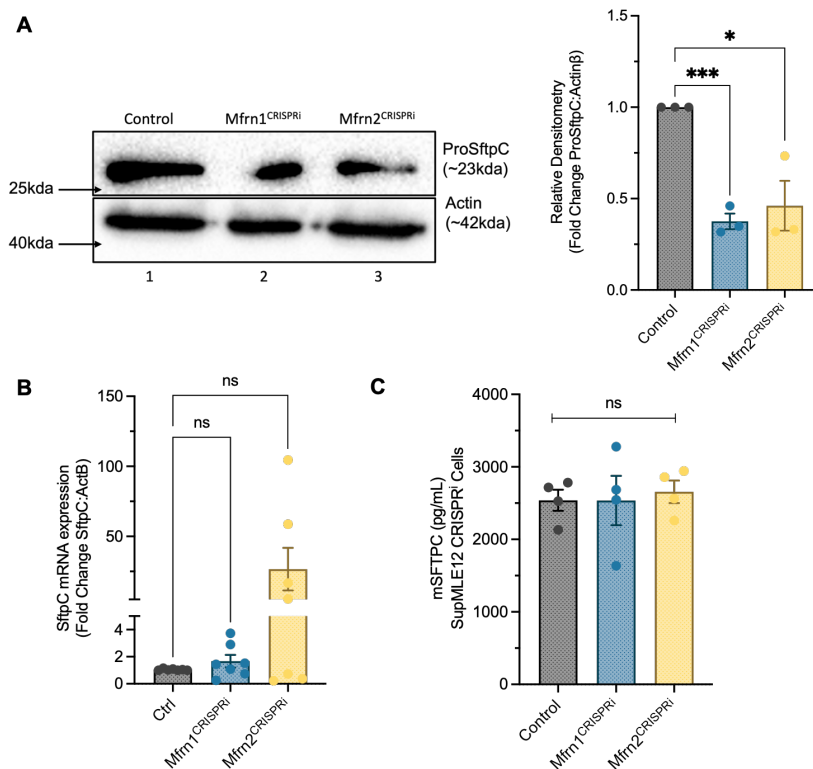


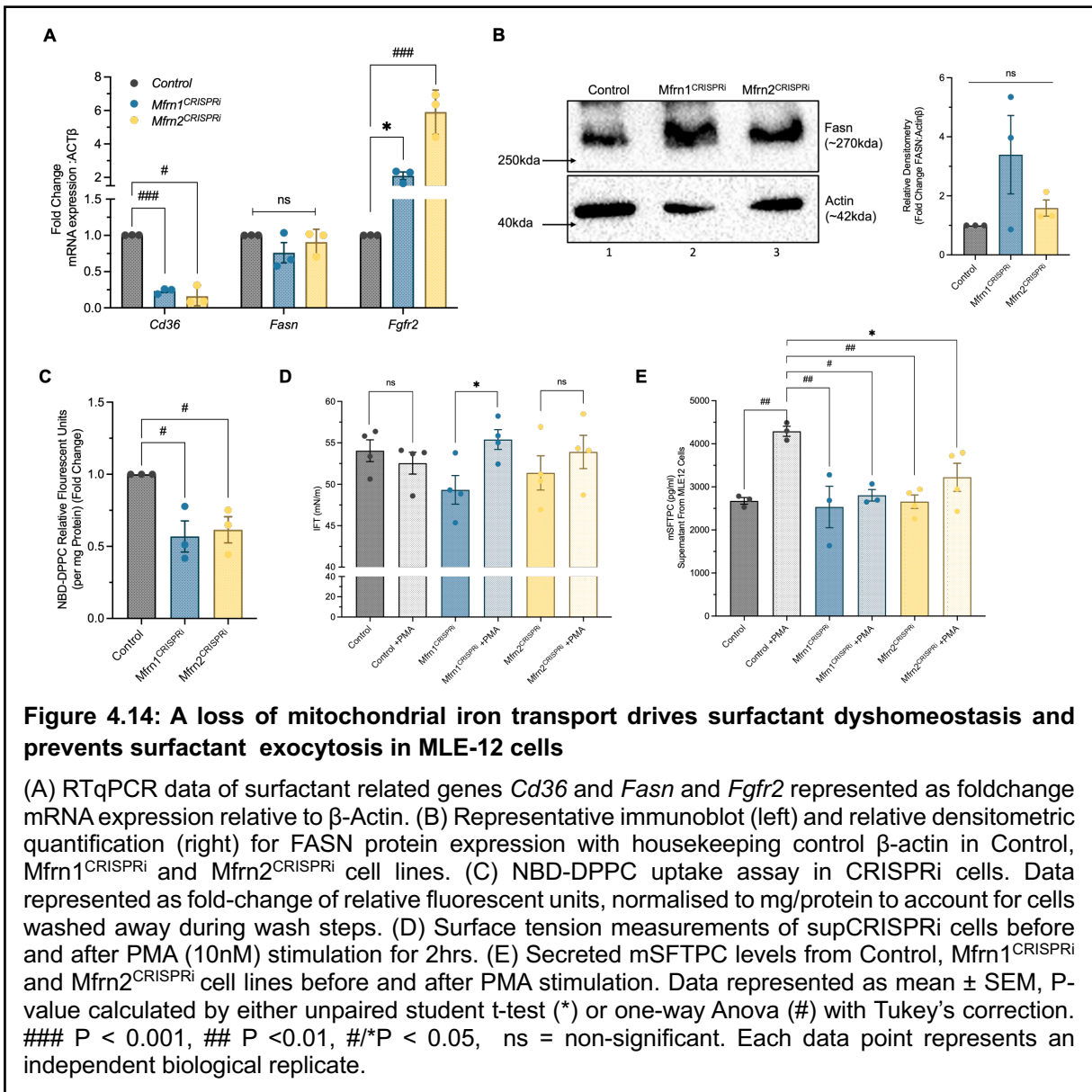
Figure 4.13: A loss of mitochondrial iron transport in MLE-12 cells perturbs Pro-SFTPC synthesis

(A) Representative immunoblot (left) and relative densitometric quantification (right) for AT2 cell functional marker Pro-SFTPC with housekeeping control β -actin in Control, *Mfrn1*^{CRISPRi} and *Mfrn2*^{CRISPRi} cell lines. (B) Gene expression of *Sftpc* in CRISPRi cells measured by RTqPCR. (C) Secreted mSFTPC levels from Control, *Mfrn1*^{CRISPRi} and *Mfrn2*^{CRISPRi} measured by ELISA. Data represented as raw values pg/ml. All data represented as mean $\pm$ SEM unless stated otherwise. P-value calculated but unpaired student t-test. *P < 0.05, ns = non-significant. Each data point represents an independent biological replicate.

Fgfr2, crucial for maintaining alveolar integrity and AT2 cell fate (73), was significantly upregulated in both *Mfrn1*^{CRISPRi} and *Mfrn2*^{CRISPRi} cells, with *Mfrn2*^{CRISPRi} showing a 6-fold increase (**Figure 4.14A**), suggesting a potential signalling response to disrupted MITOIRON

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transport. Further investigations into surfactant-related components showed a significant reduction in *Cd36* transcription (**Figure 4.14A**), a membrane protein involved in the uptake of long-chain fatty acids (FAs) (273). Although *FASN* transcription was unaffected, protein levels showed a trended increase, indicating a possible adaptive response to compensate for impaired FA import (**Figure 4.14A and 4.14B**). The NBD-DPPC uptake assay supported this hypothesis, showing significantly reduced DPPC uptake in both *Mfrn1*^{CRISPRi} and *Mfrn2*^{CRISPRi} when compared to controls (**Figure 4.14C**).



These findings suggest that impaired MITOIRON transport reduces the production and uptake of essential surfactant components, including proteins and lipid precursors. To determine whether this disruption impacts the capacity of MLE-12 cells to secrete surfactant, we stimulated cells with the secretagogue PMA and as expected in the control group we observed a trend of decreased surface tension (IFT) in the supernatant (**Figure 4.14D**). These reduced

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IFT levels were associated with an increased secretion of mSFTPC in the supernatant as well (**Figure 4.13E**). PMA stimulation failed to lower the surface tension or mSFTPC secretion in both *Mfrn1*^{CRISPRi} and *Mfrn2*^{CRISPRi} cell lines when compared to their respective controls (**Figure 4.14D and 4.14E**). Notably, *Mfrn1*^{CRISPRi} cells showed increased surface tension upon stimulation (**Figure 4.14D**). Overall, these results indicate that loss of mitochondrial iron transport disrupts MLE-12 cell function, impairing all aspects of surfactant maintenance, including uptake, production, and secretion.

4.2.4.2 A loss of mitochondrial iron transport reduces MLE-12 cell wound healing capacity

Wound healing assays on MLE-12 cells with transcriptionally silenced *Mfrn1* or *Mfrn2* revealed that loss of either transporter significantly impaired cell migration and wound closure (**Figure 4.15A and 4.15B**). Notably, *Mfrn2*^{CRISPRi} cells showed a greater impairment in wound healing capacity compared to *Mfrn1*^{CRISPRi} cells, suggesting that *Mfrn2* may play a more critical role in supporting MLE-12 cell proliferation and repair (**Figure 4.15B**). However, even *Mfrn1*^{CRISPRi} cells failed to completely close the wound by 48 hours, indicating residual functional deficits. In contrast, *Mfrn2*^{CRISPRi} deficiency was profoundly detrimental, with these cells exhibiting reduced migration and slower proliferation across all time points (**Figure 4.15A and 4.15B**).

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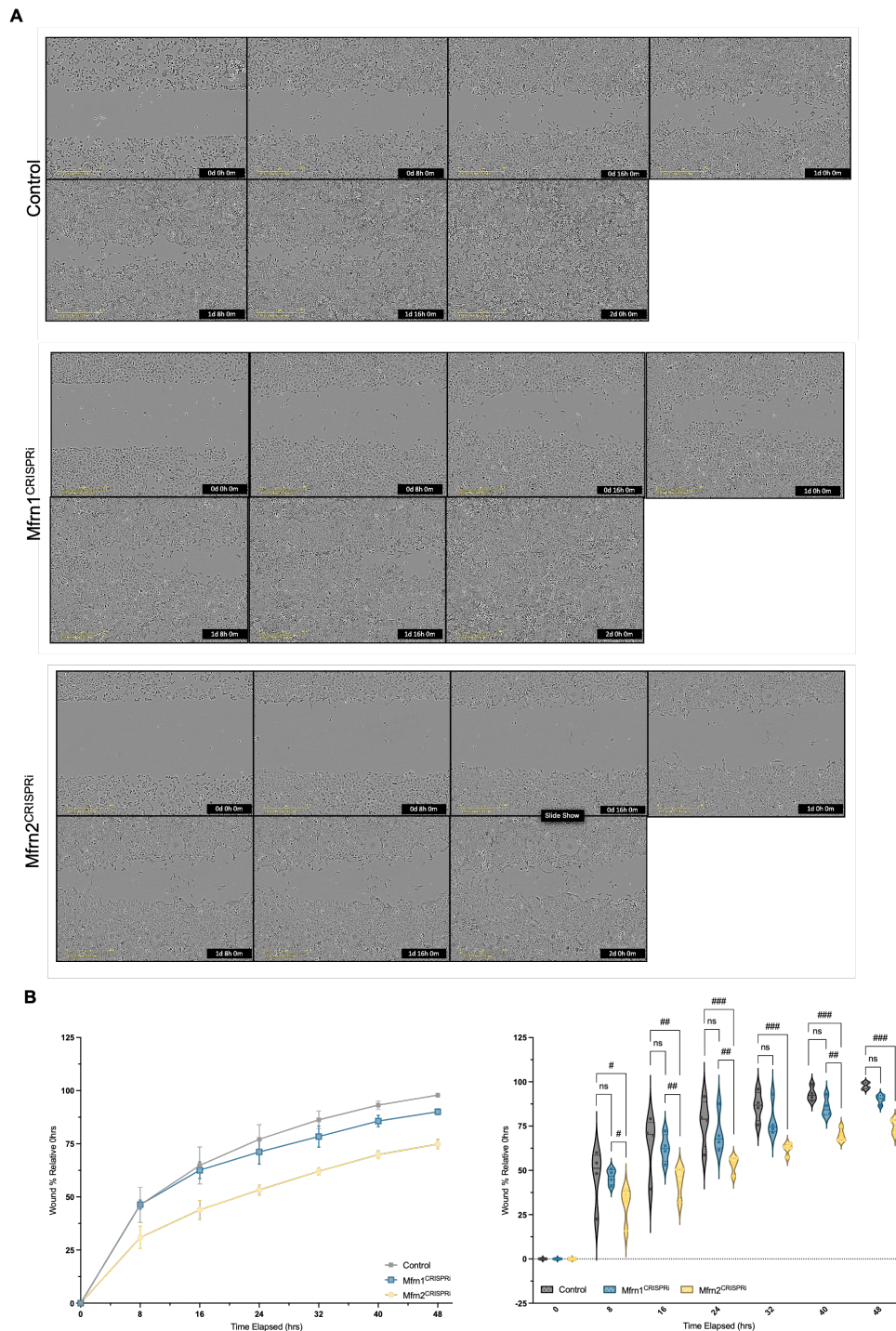


Figure 4.15: MLE-12 cells harbouring a loss of Mfrn1 or Mfrn2 have a significantly reduced wound healing capacity

(A) Representative images of Control (top), Mfrn1^{CRISPRi} (middle) and Mfrn2^{CRISPRi} (bottom) cells subjected to a scratch performed by the Incucyte® 96-Well Woundmaker Tool. As before, images were taken even 8hrs for 48hrs. Images captured at 10X. (B) Graphical representation illustrating the kinetic movement of cells and wound closure over time utilising the Satrious Essenbio software, as described previously (left). Data represented as mean $\pm$ SEM, $n=4$. Grouped analysis to evaluate statistical significance between the treatment groups at the given times ($n=3$) (right). Data represented as violin plot, ### $P < 0.001$, #### $P < 0.0001$ ns = non-significant, by two-way ANOVA with Tukey's correction.

4.2.5 AT2 Cells Alter Their Iron Metabolism When Differentiating to AT1 Cells *Ex Vivo*

Primary murine AT2 cells exhibit dynamic changes in iron-related gene expression when cultured *ex vivo* (**Figure 4.16A**). Using an AT2-to-AT1 differentiation protocol (**Chapter 3**), we demonstrate that AT2 cells at day 5 of differentiation significantly upregulate iron storage (*Fth1*, *Ftl*) and the iron export (*Fpn*) genes (**Figure 4.16A**). This surge in gene expression coincides with the peak expression of the transitional cell marker *Krt8* and then subsides at day 7 when AT1 markers are present (**Chapter 3, Figure 3.6B**). These findings suggest that AT2 cells transiently modulate their iron transcriptome to support AT2-to-AT1 transdifferentiation.

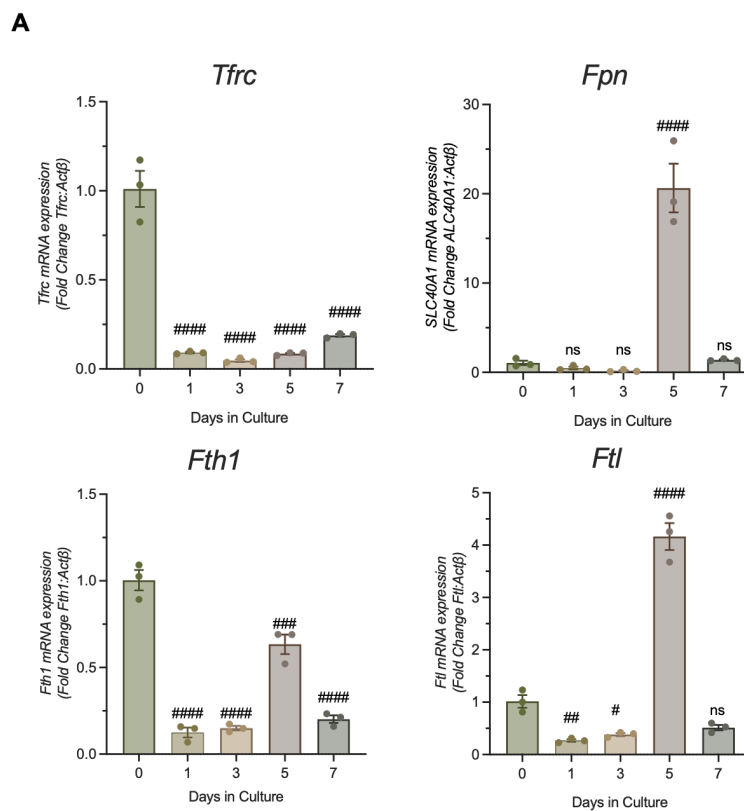


Figure 4.16: AT2 cells transition to AT1 cells upregulate iron export and iron storage regulators

(A) Gene expression analysis in CD45(-)EPCAM(+) cell population isolated from three individual mice pooled together for previously described transdifferentiation protocol (Chapter 2 and Chapter 3). RTqPCR data for iron uptake (*TfrC*), storage (*Fth1* and *Ftl*) and export (*Fpn*). Data represented as mean $\pm$ SEM, #####P<0.0001, ####P < 0.001, ##### P<0.0001 ns = non-significant, by two-way ANOVA with Tukey's correction. (n=3 technical replicates).

4.3 Discussion

Iron is essential for all cells, playing established roles in various cellular functions, including oxygen transport, DNA synthesis, and electron transport (167). As discussed in **Chapter 3**, AT2 cells are crucial for alveolar health and lung homeostasis. However, whether iron specifically contributes to AT2 cell functions, such as surfactant maintenance and self-renewal, remains poorly understood, highlighting the need for further investigation. Our findings reveal that intracellular iron levels are critical for AT2 cells, requiring a precise balance for effective function *in vitro*. Under iron-loading conditions, MLE-12 cells show impaired surfactant protein C production, reduced surfactant uptake, and decreased wound-healing capacity. Iron depletion, via extracellular chelation or CRISPRi-Cas9-mediated knockout of mitochondrial iron transporters, also disrupts surfactant secretion, uptake, and production, while also impairing wound healing. These results underscore the intricate dependence of AT2-specific functions on tightly regulated iron homeostasis.

4.3.1 Iron Regulation in MLE-12 Cells

Under iron-replete conditions, AT2-like cells downregulate iron uptake while increasing iron export and storage machinery, consistent with an adaptive cellular response to elevated iron levels (143). Treatment with the extracellular iron chelator DFX induces the opposite effect, enhancing iron uptake and reducing export to counteract intracellular iron depletion, reflecting their intrinsic iron regulatory mechanisms. Interestingly, the intracellular iron chelator DFP, despite reducing intracellular iron levels to a similar extent as DFX, does not elicit comparable upregulation of iron regulatory pathways. This suggests that MLE-12 cells may not sense iron deficiency effectively in the presence of DFP, possibly because DFP binds and redistributes intracellular iron, either shuttling it to organelles or exporting it out of the cell, preventing true iron deficiency (118, 272). Additionally, differences in chelation efficiency may play a role. DFP binds iron in a 3:1 (bidentate) complex, whereas DFX forms 1:1 (hexadentate) complexes (272). At equal concentrations, DFX may chelate iron more rapidly, inducing deficiency more effectively, while DFP chelates and redistributes iron more gradually amongst organelles, potentially delaying cellular sensing of iron loss. Further investigation into the kinetics of the different chelators and the corresponding adjustments of iron regulatory proteins is needed to clarify these observations.

Mitochondria house 20–50% of cellular iron, making them critical hubs for iron metabolism. With this in mind, we established a model of dysregulated iron metabolism by targeting the mitochondrial iron transporters *Mfrn1* or *Mfrn2*. Consistent with previous findings, loss of either transporter significantly reduced MITOIRON, total cellular iron, and heme levels (149-152, 156). Furthermore, our findings demonstrate that mitochondrial iron transport is essential for

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MLE-12 cell proliferation and survival. In response to cellular and mitochondrial iron deficiency, CRISPRi cells adjust their iron metabolism by upregulating iron uptake and downregulating export pathways, consistent with an adaptive response to maintain iron availability (143). Interestingly, silencing different mitochondrial iron transporters appears to differentially impact iron storage pathways. Under iron-deplete conditions, ferritin heavy chain expression is typically downregulated to mobilize stored iron (143), as observed in *Mfrn1*^{CRISPRi} cell line and the DFX model. On the other hand, *Mfrn2*^{CRISPRi} cells fail to decrease FTH effectively, implying a reduced capacity to mobilize stored iron. These findings may suggest that *Mfrn2* may play a more prominent role in cellular iron sensing and regulation in AT2-like MLE-12 cells, aligning with its proposed function in maintaining mitochondrial iron homeostasis in non-erythroid cells (152, 156). However, further studies are needed to clarify each transporter's role and the implications this has for AT2 cellular iron metabolism.

4.3.2 The Impact of Iron on AT2 Cell Specific Functions *In Vitro*.

4.3.2.1 Surfactant maintenance

The interplay between iron metabolism and surfactant homeostasis in AT2 cells remains poorly understood. Our findings reveal that both iron-replete and iron-deplete conditions disrupt surfactant homeostasis, with iron depletion exerting more profound effects. Iron repletion perturbs iron uptake pathways and alters the turnover and synthesis of surfactant protein C. In contrast, iron depletion, induced by extracellular chelation (DFX) or CRISPRi-mediated loss of mitochondrial iron transport, disrupts multiple facets of surfactant homeostasis, including uptake, turnover, synthesis, and secretion. These observations underscore the necessity of maintaining stable intracellular iron levels, as both deficiency and excess impair MLE-12 cells capacity to maintain functional surfactant.

Stimulation of surfactant secretion with PMA led to a two-fold increase in intracellular iron levels at 2 hours, which returned to baseline by 3 hours. This temporal pattern suggests that coordinated iron influx, utilisation and efflux may support surfactant secretion. In biology, AT2 cells have been shown to rely heavily on calcium influx and intracellular stores to facilitate surfactant exocytosis (3, 274). Whether iron plays a similar role remains unknown. However, evidence from other cell types suggests significant crosstalk between iron and calcium metabolism, with cytosolic Ca²⁺ modulating iron entry in astrocytes, and the MCU facilitating Fe²⁺ transport under specific conditions (159, 275, 276). Furthermore, calcium channel blockers have demonstrated a decrease in cellular iron absorption (276). Taken together it is

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possible the observed increase in intracellular iron levels may be driven by elevated Ca^{2+} demand during exocytosis or by MCU-mediated co-transport of iron alongside calcium.

The observed increase in iron uptake machinery and intracellular iron levels upon PMA stimulation may partially result from off-target effects of PMA. PMA is classically used to activate immune cells, triggering cascades of signalling pathways, including inflammatory pathways closely linked to iron metabolism (277). Additionally, high concentrations of PMA enhance transferrin receptor activity, leading to increased iron uptake depending on the cell type (262). These potential off-target effects cannot be overlooked, underscoring the need for future studies employing alternative secretagogues to clarify the specific role of iron in surfactant secretion and to minimize confounding influences of PMA.

Despite these considerations, our findings show that iron-deficient MLE-12 cells fail to secrete surfactant upon PMA stimulation, highlighting a consistent essential role for intracellular iron bio-availability in surfactant secretion. Iron deprivation through extracellular chelation (DFX) inhibited iron uptake, while intracellular chelation (DFP) disrupted iron utilization. A loss of MITOIRON transport impaired both uptake and utilization. Collectively, these results confirm the indispensable role of iron in surfactant exocytosis, emphasizing the critical importance of iron uptake, intracellular iron bio-availability and MITOIRON metabolism in maintaining surfactant homeostasis.

A plausible explanation for the requirement of iron uptake and mitochondrial iron import lies in the critical role iron plays in mitochondrial function and metabolism. AT2 cells, known for their high energy demands, possess an abundance of mitochondria residing closer to the nucleus to support these needs (68). Surfactant secretion is an energy-intensive process that relies heavily on ATP, which has been demonstrated to stimulate surfactant secretion in AT2 cells (24, 274). Iron is essential for ATP production, as it is required for the biogenesis of Fe-S clusters and the activity of key enzymes such as ACO2 and SDH within the TCA cycle (162, 167). Additionally, the ETC complexes critical for OXPHOS depend on iron-containing co-factors (162, 167). Consequently, iron deficiency *in vitro* reduces ATP production, with iron chelation impairing the TCA cycle (278). We demonstrate that a loss of mitochondrial iron transport disrupts heme biosynthesis, a key function of the mitochondria. Although ATP production and other elements of the TCA cycle were not directly assessed, it is likely that both are impaired, disrupting cellular energy production and other metabolic pathways critical for surfactant exocytosis in MLE-12 cells.

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An additional arm in which iron could be affecting surfactant is through its intricate relationship with lipid metabolism (167). Surfactant consists of ~90% lipids, with ~80% being phospholipids, derived either from recycled/remodelled lipids or *de novo* synthesis (3, 13). Indirectly examining recycling through the previously described surfactant uptake assay in **Chapter 3**, iron chelation does not appear to affect surfactant lipid uptake in MLE-12 cells. Iron replete conditions on the other hand significantly reduce the uptake of NBD-DPPC. Previous studies have highlighted the detrimental effects of non-protein-bound iron (free iron), which acts as a catalyst for the peroxidation of pulmonary surfactant lipids function (268, 279). Therefore, the limitation of lipid uptake under iron-replete conditions could be a protective mechanism to avoid lipid peroxidation, ferroptosis and ultimately cell death.

A loss of mitochondrial iron transport also markedly impairs surfactant lipid uptake. Further investigations highlighted a significant decreased expression in *Cd36*, a key scavenger receptor for FA's including DPPC (280). These results would suggest that a loss of mitochondrial iron transport may directly impact lipid surfactant uptake by affecting the expression of their receptors. Investigating the uptake of other surfactant lipids and whether this effect extends beyond CD36 could provide further insights into the broader impact of mitochondrial iron transport on lipid uptake in AT2 cells.

Under iron-replete conditions, SFTP-C synthesis and processing are disrupted, as evidenced by a marked reduction in both the pro-protein and mature forms. Interestingly, *Sftpc* transcript levels are significantly upregulated, suggesting a compensatory transcriptional response to impaired protein maturation or stability. Additionally, lipid biosynthesis (FASN expression) and other surfactant-related genes show no significant increase, suggesting *de novo* FA synthesis is not activated. As described above, this may serve to mitigate the potential toxicity of excess iron on surfactant lipids (268, 279). Consistently, AT2 cells from FPN-mutant mice, which exhibit alveolar iron overload, show altered surfactant storage, underscoring the impact increased iron levels have on surfactant homeostasis (188). However, it remains unclear whether iron levels are directly elevated in AT2 cells of these mice, preventing direct comparisons.

MITOIRON transport correlates with increased FASN protein levels and a ~2-fold upregulation of *Fgfr2* expression. FASN is stimulated by FGF7, a fibroblast-secreted ligand taken up by FGFR2 in AT2 cells to drive surfactant production (252). Although this *in vitro* model lacks AT2-fibroblast crosstalk, the concurrent upregulation of *Fgfr2* RNA and FASN protein suggests *de*

novo synthesis may be employed to compensate for impaired lipid uptake caused by a loss of mitochondrial iron transport.

4.3.2.2 Repair capacity

Iron plays a well-established role in cellular proliferation and growth; however, its specific function in AT2 cell repair and regeneration remains underexplored. Using a wound healing assay (described in **Chapter 3**), we demonstrate that intracellular iron levels directly influence MLE-12 cell repair capacity. Iron repletion significantly impairs cell migration after 48 hours. Similarly, iron deficient MLE-12 cells, either through extracellular chelation or loss of mitochondrial iron transport, has an even more pronounced effect on wound healing. In contrast, intracellular iron chelation via DFP enhances proliferation and accelerates recovery when compared to control cells. These findings suggest that maintaining optimal intracellular iron balance is crucial for MLE-12 cell proliferation and repair.

Iron deprivation is reported to induce G1/S phase arrest, thus inhibiting cell cycle progression (281). This growth-limiting effect has been leveraged in cancer therapy, with recent studies demonstrating reduced alveolar organoid and tumour sphere formation in primary mouse AT2 cells (267, 281). Given this, it is not surprising that extracellular iron chelation significantly impairs wound healing in MLE-12 cells. In contrast, intracellular iron chelation via DFP appears to enhance proliferation and promote recovery post-injury. This may be attributed to DFP's ability to bind and redistribute intracellular iron without triggering the IRP-IRE signalling system, as iron storage, uptake, and export remain unchanged (118, 272). A similar phenomenon has been observed in tumour alveolar spheres treated with the iron-trapping siderophore 2,5-dihydroxybenzoic acid, which significantly reduces labile iron levels while promoting sphere formation (267). Like DFP, this siderophore is thought to facilitate iron shuttling in and out of cells (282). In fibroblasts, low-dose DFP treatment has been shown to enhance proliferation and metabolism (283). This effect is associated with increased HIF-1 α protein levels, presumably due to chelation of cytosolic Fe²⁺ required for PHD enzyme activity, leading to HIF-1 α stabilisation (283). In turn, this may activate hypoxia-responsive pathways that promote cell migration and reduce iron-catalysed oxidative stress. In contrast, DFX predominantly chelates extracellular iron, failing to trigger similar intracellular responses. Whether this mechanism explains the enhanced wound healing observed in DFP treated MLE-12 cells remains to be determined.

In chronic lung diseases, AT2 cells often lose their regenerative potential, failing to repair the damaged epithelium (8). Iron dysregulation is a well-documented feature of these diseases,

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with some studies suggesting that iron accumulation in AT2 cells contributes to their dysfunction (182, 284-286). Furthermore, MLE-12 cells treated with erastin, which induces iron accumulation, led to inhibition of cell proliferation and increased cell death (287). In agreement, our findings suggest that excess iron negatively impacts MLE-12 cells repair functions. Although this model does not mimic chronic iron overload seen in disease, the cells experience significant iron loading. Notably, the cells still appear to replicate, evidenced by increased confluency, yet they fail to migrate and close the wound, indicating that excessive iron may disrupt their migratory capacity of these cells. Conversely, systemic iron overload due to ferroportin mutations, which leads to lung iron accumulation, was associated with both AT2 cell hyperplasia and hypotrophy (188). This balance suggests that iron overload in this context does not directly impair AT2 cell repair function.

Recent studies reveal that aged AT2 cells develop functional iron deficiency, impairing stem cell function and inhibiting tumour development (267). The NUPR1-LCN2 axis contributes to this iron insufficiency, suppressing AT2 to AT1 cell differentiation, with iron supplementation restoring differentiation. Disrupting the NUPR1-LCN2 axis via intratracheal gRNA instillation, a non-cell-specific method, leaves it unclear whether AT2 or neighbouring cells drive impaired stem cell capacity. Furthermore, this iron insufficiency may stem from conditions of the tumour microenvironment. However, the study consistently highlights AT2 cell dependence on iron for survival and proliferation. Consistent with this, *ex vivo* cultures of isolated mouse AT2 cells show a significant increase in iron-related genes as they transition to AT1 cells. Investigating into whether iron supplementation or chelation accelerates or disrupts this process would provide more insight.

4.4 Limitations

In this chapter, we establish a role for iron in AT2-specific functions, including surfactant maintenance and repair capacity. However, several limitations must be considered when drawing definitive conclusions from these findings.

A key limitation is that all experiments, excluding the AT2-AT1 protocol, were conducted using the murine AT2-like cell line MLE-12. As discussed in **Chapter 3**, MLE-12 cells are reportedly the most similar to primary AT2 cells, particularly in their ability to actively secrete surfactant lipids upon stimulation, a function not reported in other AT2-like cell lines (**Figure 3.1**) (244, 261). Investigating iron dynamics in additional AT2-like cell lines or *ex vivo* primary and organoid models would provide deeper insights into iron's role in surfactant secretion, synthesis, and turnover. Additionally, surfactant secretion in our model was synthetically induced using a well-documented secretagogue (3, 223), which as discussed has reported off-target effects (277). In contrast, *in vivo* surfactant production is influenced by complex physiological factors, including paracrine signalling from AT1 cells and mechanical stretch during respiration, neither of which are replicated in this study (3, 24, 274). Thus, it remains unclear whether AT2 cells exhibit the same iron dependency under physiological conditions.

Regarding repair capacity, a key limitation is that MLE-12 cells are a transformed cell line, meaning they have a higher baseline proliferative capacity compared to primary AT2 cells (244). This makes it difficult to directly translate findings related to their transdifferentiation potential. Reproducing these assays in primary AT2 cells would strengthen the findings discussed above. Furthermore, *in vivo* AT2 cell repair is significantly influenced by environmental factors (54, 73, 288, 289). Including AT1 cell death and cross-talk with other cell types, particularly fibroblasts, which activate signalling cascades driving self-renewal and transdifferentiation into AT1 cells (73, 288). Since this study was conducted under artificial conditions, it does not fully recapitulate the complexity of *in vivo* repair processes.

Another limitation pertains to the AT2-to-AT1 transdifferentiation protocol. Although we observed striking modulation of iron-related genes, these findings must be validated through additional experiments. The major challenge with this protocol is the requirement for a large number of mice for cell numbers, which presents ethical and practical limitations. Future studies could leverage publicly available datasets on AT2-to-AT1 differentiation. Investigating whether a specific iron-regulatory profile emerges during this process could provide further mechanistic insights, though such datasets have not been established to date.

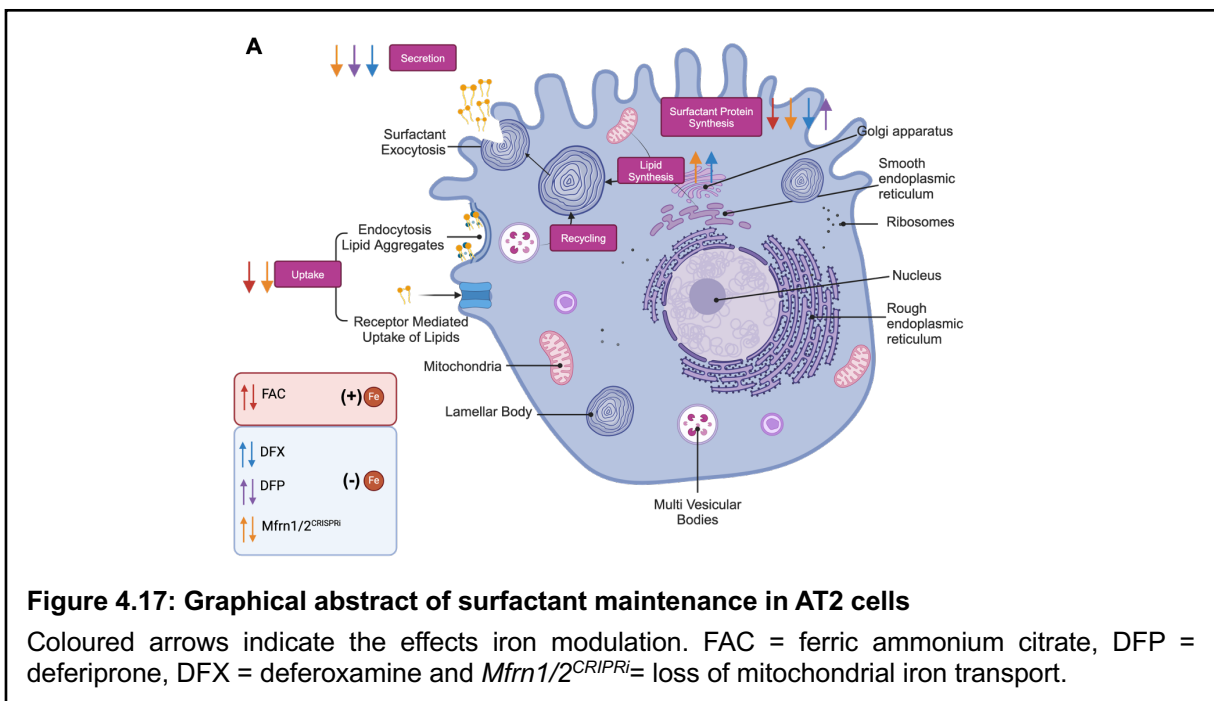
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Finally, an important limitation of the CRISPRi cell lines utilized in this study is the insufficient characterization of potential off-target effects related to the specificity of the mitoferrin knock-down. During initial cell line generation, several clones were screened for efficient knockdown of Mitoferrin 1 or 2, but off-target effects were not thoroughly evaluated. Ultimately, a single clone was selected for further experiments assessing impaired mitochondrial iron transport in MLE-12 cells. This reliance on a single clone restricts the interpretation of results, as observed phenotypes could potentially arise from clonal artifacts rather than from specific Mitoferrin deficiency. Future studies should therefore include comprehensive off-target analyses and utilize multiple independent clones or rescue experiments to robustly validate the observed cellular phenotypes.

4.5 Conclusions

Our findings underscore the critical role of iron homeostasis in AT2 cell function, demonstrating that both iron excess and deficiency differentially disrupt key processes such as surfactant metabolism and epithelial repair (**Figure 17A**). By modulating iron levels in MLE-12 cells and leveraging our AT2-specific tool-kit outlined in **Chapter 3**, we show that intracellular iron availability is essential for maintaining AT2-specific functions. Notably, the loss of MITOIRON transport exerts the most pronounced effects, leading to impaired surfactant secretion, disrupted lipid uptake, and decreased wound healing capacity. Extracellular iron chelation partially recapitulates these defects, further positioning iron as a central metabolic regulator of AT2 cell specific functions.

Differentiation studies in primary murine AT2 cells further reveal that AT2 cells dynamically modulate their iron transcriptome to facilitate AT2-to-AT1 transition, implicating iron metabolism in alveolar epithelial renewal. Collectively, these findings highlight the necessity of tightly regulated iron homeostasis for maintaining AT2-specific functions. By elucidating the mechanisms through which iron governs surfactant production, lipid metabolism, and epithelial repair, this study provides a foundation for future research into the broader implications of iron dysregulation in AT2 cells and its role in lung health and disease.



5. Expanding the Role of Iron in AT2 Cell Function *In Vivo*: Implications for Lung Health

5.1 Introduction

For the first time, **Chapter 4** demonstrated the fundamental role of iron in AT2-specific functions *in vitro*, highlighting its importance in surfactant homeostasis and AT2-repair. Here, we extend this investigation *in vivo*, examining how a loss in mitochondrial iron transport affects AT2 function and how AT2-specific iron dysregulation shapes the alveolar microenvironment and impacts lung health. Given their key role in lung health, understanding molecular drivers, like iron metabolism, in AT2 function and dysfunction is crucial for advancing lung research.

Lung iron regulation is an emerging but understudied area of research, with most studies focusing on lung iron homeostasis by altering systemic iron metabolism (182, 188, 189, 290). The effects of increased pulmonary iron depend on the mechanism of elevation. Disruption of the ferroportin/hepcidin axis leads to alveolar iron accumulation and restrictive lung disease, characterized by air-blood barrier thickening and balanced AT2 cell hyperplasia and hypertrophy (188, 189). In Transferrin2-mutant and hemochromatosis protein-deficient mice, increased pulmonary iron is associated with small airway fibrosis with no reported disruptions to AT2 cell health (115). Conversely, intratracheal iron administration (Fe^{3+} or Fe^{2+}) induces acute lung injury and fibrosis (119, 268). The discrepancies between these models likely arise from the difference between acute and chronic iron overload. In FPN-mutant mice, tolerable iron levels did not impair AT2 cells. Similarly, in Ali *et al.* (2020), while iron levels gradually increased in mutant mice, compensatory mechanisms likely helped mitigate fibrosis and damage by safely binding and storing iron. However, direct iron instillation overwhelms these binding proteins, leading to epithelial damage, acute lung injury and fibrosis (268, 269).

Elevated systemic and pulmonary iron levels are observed in both human and experimental models of restrictive lung diseases [2, 9, 10]. Recently, AT2 cells were characterized as having an iron accumulation signature in IPF [9]. With previous studies suggesting that iron localizes to AT2 cells in IPF, promoting their dysfunction and driving cell death pathways [10]. Of note, these studies often focus on late-stage fibrosis with established iron dysregulation. Acute and chronic experimental models of COPD have correlated CS-induced AT2 cell injury to ferroptosis, an iron dependent cell death pathway (209, 210). Similarly, both studies highlight ferroptosis signatures once injury and inflammation are established, therefore it remains unclear whether AT2 dysfunction drives iron deposition or results from it in both experimental models of COPD and fibrosis warranting further investigations.

Given the strong link between lung iron over-load and lung disease, iron chelation has been considered as a potential therapeutic in several chronic lung diseases. Mitochondrial iron chelation ameliorates cigarette smoke-induced bronchitis and emphysema *in vivo* (118). Similar is seen for murine models of allergic asthma and BLM-induced fibrosis with iron-chelating complexes reducing lung pathogenesis (115, 208, 291). This suggests that iron overload is an important factor driving disease pathogenesis in these models. Paradoxically, systemic iron deficiency worsens smoke-induced inflammation and emphysema (290). Whether this stems from local or global iron deficiency remains unclear. However, targeted airway hepcidin silencing, reduces FPN degradation and induces cellular iron deficiency in AMs, exacerbates sepsis-induced lung injury *in vivo* (194). Together this suggests that local, cellular-level iron deficiency can be detrimental to the lungs response to injury.

In systemic iron overload mouse models, the effects of lung iron accumulation and response to BLM-induced fibrosis remains debated (115, 292). Tfr2-mutant mice intrathecally instilled with BLM presented with an increased Ashcroft score, a histological measure of lung fibrosis, alongside elevated baseline collagen deposition (115). Of note, this study assessed fibrosis severity primarily through collagen deposition surrounding small airways. In contrast, systemic iron overload in hepcidin-knockout mice on a high-iron diet had no effect on bleomycin-induced fibrosis (292). These discrepancies likely stem from differences in genetic and dietary models. However, the findings from Zhang *et al.* (2020) suggest that iron overload may be a consequence rather than a driver of fibrosis.

To date, no studies have specifically manipulated iron in AT2 cells to assess its impact on AT2-cell specific functions and response to injury. Targeted gene deletions in AT2 cells have been shown to impair their function and disrupt overall lung health, reinforcing their central role in alveolar homeostasis (81, 258, 293). Studies show that AT2-specific gene deletions can increase susceptibility to inflammation and fibrosis, driving immune cell infiltration into the alveolar space (81, 258, 293). For example, loss of *Slc38a8*, a zinc transporter, impaired AT2 recovery and led to spontaneous fibrosis in aged mice, while deletion of mitochondrial genes *Mitofusin 1* and *Mitofusin 2* disrupted lipid metabolism, triggering spontaneous lung fibrosis (81, 258). Interestingly, *in vitro* studies haven also characterized *Slc38a8* as an iron transporter (137). These findings highlight the importance of mitochondrial function and nutrient availability in AT2 cell homeostasis. Given the results from **Chapter 4**, we hypothesize that impaired MITOIRON transport in AT2 cells *in-vivo* will further compromise their function by disrupting nutrient availability and mitochondrial integrity. Additionally, given the broader

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impact of AT2 dysfunction on the alveolar microenvironment, we further propose that loss of mitochondrial iron transport will contribute to lung inflammation and injury.

5.1.1 Chapter Aims and Objectives

Despite efforts to understand iron and MITOIRON regulation in the lung, its direct impact on AT2 cell function remains largely unexplored. Existing studies have used systemic iron manipulation or examined iron dynamics in lung pathology to elucidate lung-iron regulatory pathways. However, these studies lack specificity, with global inhibitors masking which organs or cell types are most vulnerable to iron dysregulation. While AT2-specific knockout studies have shown that mitochondrial function and nutrient availability are crucial for AT2 cell health, no study has specifically targeted iron metabolism in AT2 cells. This highlights the need for more targeted approaches to understand the role of iron in AT2 cell function.

Building on the results from **Chapter 4**, this chapter aims to address these knowledge gaps by establishing an AT2 cell-specific model of MITOIRON dysregulation and assess its impact on AT2-specific function, alveolar homeostasis, and lung health. Additionally, we will explore AT2 cell - AM interactions under normal and iron-deficient conditions *in vitro*, to determine how AT2-derived signals influence immune cell function. By underpinning the role of iron in AT2 cell health, this work will provide fundamental insights into AT2 cell function potentially unveiling mechanisms by which iron governs lung health and dysfunction in chronic lung disease.

This chapter aims to;

1. Establish an *in vivo* model of dysregulated MITOIRON iron metabolism specifically in AT2 cells.
2. Examine how dysregulated MITOIRON metabolism in AT2 cells alters their function *in vivo*.
3. Investigate how a loss of MITOIRON in AT2 cells alters the alveolar microenvironment and impacts lung health.
4. Establish an *in vitro* AT2-AM cross-talk model using MLE-12 cells and foetal-derived alveolar macrophages (FLAM).
5. Define the effects both a healthy and iron deficient secretome from AT2 MLE-12 cells have on FLAM function.

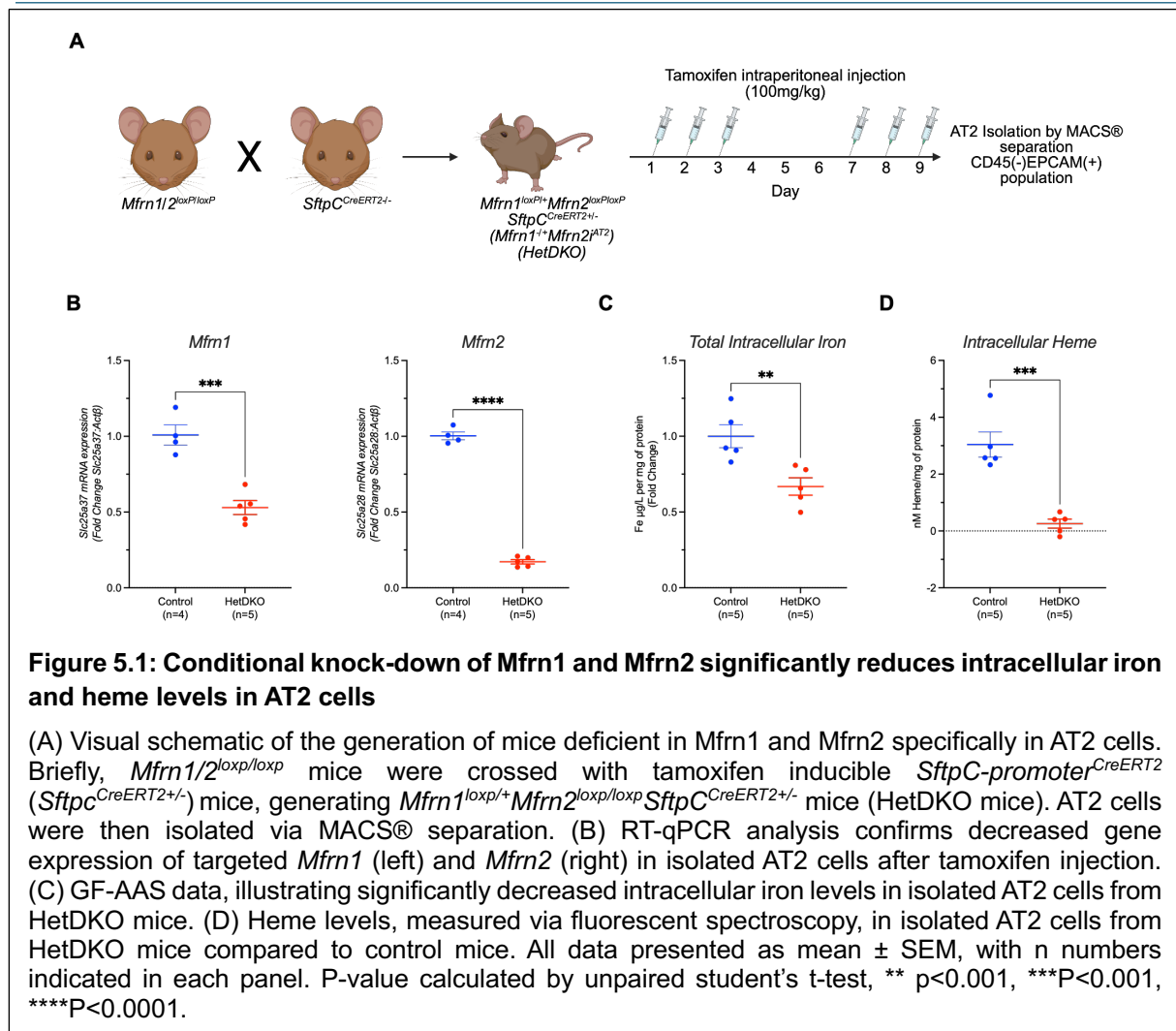
5.2 Results

Building on our initial observations in **Chapter 4** of how iron-replete and iron-deplete conditions affect AT2 cell function *in vitro*, we aimed to further explore the role of iron using an *in vivo* model with targeted dysregulation of MITOIRON metabolism specifically in AT2 cells. Additionally, we sought to elucidate the impact decreased iron levels in AT2 cells has on lung health and the alveolar microenvironment.

5.2.1 Establishing an *In Vivo* Model of Dysregulated Iron Metabolism in AT2 Cells.

Utilizing the lung-specific SFTPC promoter, we conditionally deleted *Mfrn1* and *Mfrn2* in AT2 cells (see methods, **section 2.3.1 Chapter 2**). Briefly, *Mfrn1/2^{loxP/loxP}* mice were crossed with *Sftpc^{CreERT2+/-}* mice. As both *Mfrn1* (flox) and *Sftpc* (Cre) are on chromosome 8, meiotic recombination between them is reduced, decreasing the likelihood of achieving a homozygous *Mfrn1^{loxP/loxP}* to roughly 1/500. A previous member in the lab therefore generated *Mfrn1^{loxP/+} Mfrn2^{loxP/loxP} Sftpc^{CreERT2+/-}* mice (hereafter termed HetDKO mice) (**Figure 5.1A**). Following tamoxifen (TMX) injection, AT2 cells were isolated from HetDKO mice by CD45⁻ EpCAM⁺ selection (MACS[®] separation) (**Figure 5.1A**). RT-qPCR confirmed the reduced expression of *Mfrn1* and *Mfrn2* in isolated AT2 cells (**Figure 5.1B**). Subsequent GF-AAS analysis demonstrated significantly lower cellular iron levels (**Figure 5.1C**), accompanied by decreased heme levels in isolated AT2 cells (**Figure 5.1D**). These findings illustrate that tamoxifen-induced conditional deletion of *Mfrn1* and *Mfrn2* in AT2 cells effectively reduces both intracellular iron and heme levels.

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5.2.1.1 A loss of mitochondrial iron transport in AT2 cells alters the expression of genes related to iron metabolism and surfactant homeostasis.

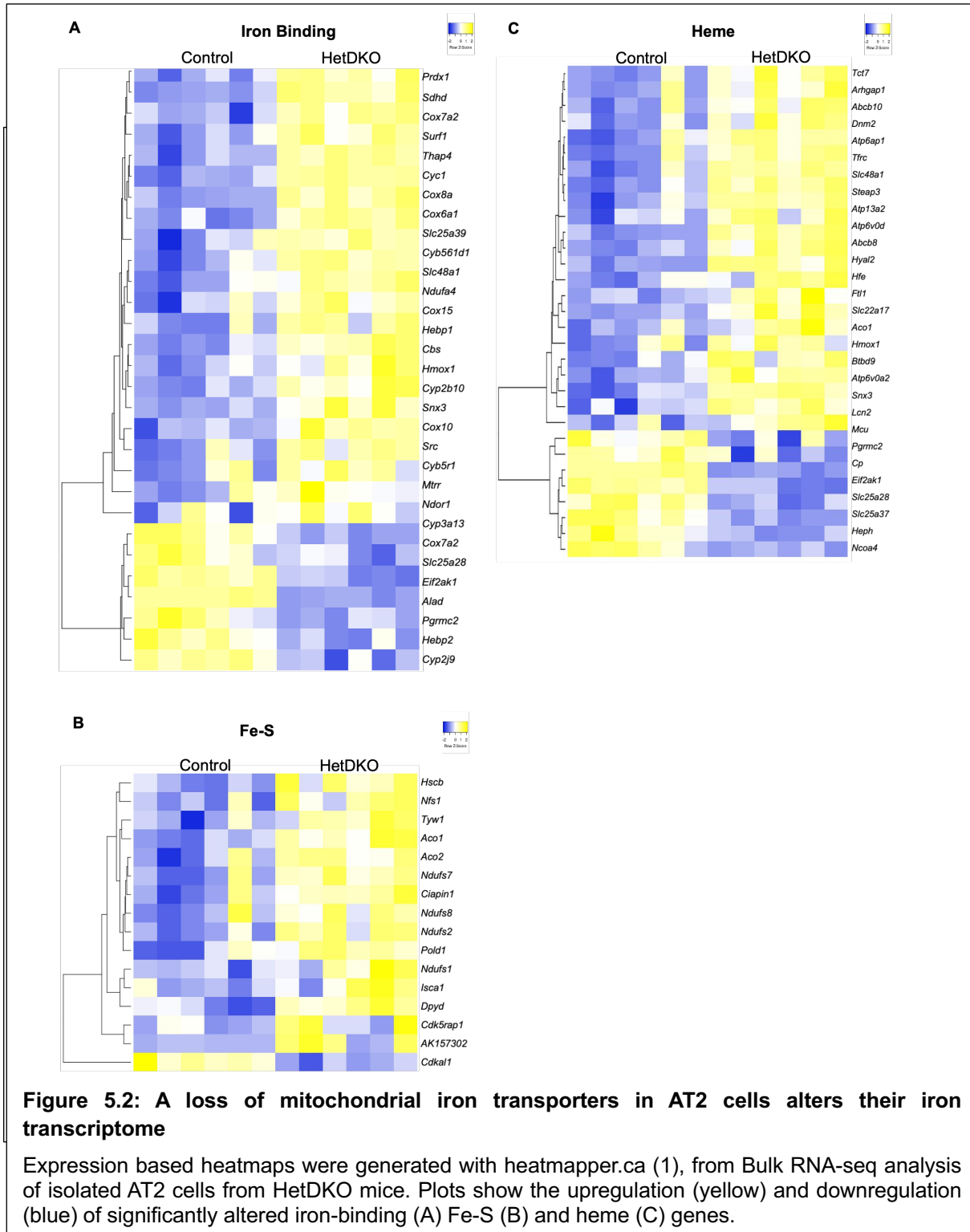
RNA-seq analysis of isolated AT2 cells from HetDKO mice revealed a significantly altered iron-related transcriptome (**Figure 5.2A – 5.2C**). Genes involved in iron uptake and transport, such as *Tfrc*, *Steap3* (TF-independent iron uptake), and *Lcn2* (iron-trafficking siderophore), were markedly upregulated. Altered expression in genes related to Fe-S cluster biogenesis (Aconitase 1 [*Aco1*], Nuclear Fe-S Cluster Assembly Factor [*Nufs*], Cytoplasmic Iron-Sulfur Assembly Protein 1 [*Ciao1*]) (**Figure 5.2B**) and heme synthesis/regulation (**Figure 5.2C**) (*HO1*, *Eif2ak1/Hri*, *Hebp1*, Solute Carrier Family 48 Member 1 [*Slc48a1*]) further indicated disrupted iron metabolism in AT2 cells.

Specifically, genes involved in mitochondrial OXPHOS were significantly upregulated, including Aconitase 1 and 2, as well as genes associated with Complex-I (*Ndufs7*, *Ndufs8*, *Ndufs2*, and *Ndufs1*) and Complex-IV (*Cox7a2*, *Cox8a*, *Cox6a1*, and *Cox15*). Genes involved in mitochondrial redox regulation, peroxidin 1 (*Prdx1*) and solute carrier family 25 member 39

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transporter (*Slc25a39*) are significantly increased (294, 295). *Eif2ak1/HRI*, a key regulator of heme homeostasis and the integrated stress response (ISR), is significantly decreased in AT2 cells with disrupted MITOIRON transport (Figure 5.2C).

Loss of *Mfrn1/2* in AT2 cells was also associated with downregulation of key surfactant homeostasis genes, including adhesion G protein-coupled receptor 5 (*Adgrf5*), coiled-coil



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domain containing 59 (*Ccdc59*), fibroblast growth factor 7 (*Fgf7*) (**Figure 5.3A**). Of note, genes involved in surfactant lipid production *Sftp-B*, *Sftp-C*, glycerophosphodiester phosphodiesterase 1 (*Gde1*), platelet-activating factor acetylhydrolase 2 (*Pafah2*) were upregulated in AT2 cells when compared to controls (**Figure 5.3A**). Furthermore, there was an upregulation in several subunits of ATP synthase which are required for ATP production in the mitochondria (*Atp5a1*, *Atp5b*, *Atp5g3* and *Atp5o*).

5.2.2 Developing Homozygous Loss of *Mfrn1* and *Mfrn2* in AT2 Cells *In Vivo*

As mentioned there was a small probability of obtaining *Mfrn1*^{-/-}*Mfrn2*^{iAT2} (hereafter termed *Mfrn1/2*^{iΔAT2} or Real DKO, RDKO) mice; a previous member in the lab, Ziling Huang successfully generated this model at Weill Cornell Medicine, New York. Homozygous loss of *Mfrn1* and *Mfrn2* in AT2 cells resulted in rapid weight loss 2-3 weeks post-tamoxifen injection and a 50-60% mortality rate (**Figure 5.4A – 5.4D**). At 3.5 weeks, lung tissue from surviving mice exhibited neutrophilic, proliferative interstitial pneumonia with hyaline membranes and mild to moderate fibrosis (**Figure 5.4E**). Additionally AT2 cell hyperplasia and neutrophilic cuffs were observed (**Figure 5.4E**). RDKO mice also showed increased cell infiltration in BALF and reduced lung function parameters (increased elastance, decreased inspiratory capacity and respiratory compliance) (**Figure 5.4F - 5.4I**). These results indicate that RDKO mice develop spontaneous ALI associated with fibrosis, inflammation, impaired lung physiology, and high morbidity and mortality, underscoring the critical role of mitochondrial iron transport, specifically in AT2 cells, for healthy alveolar function.

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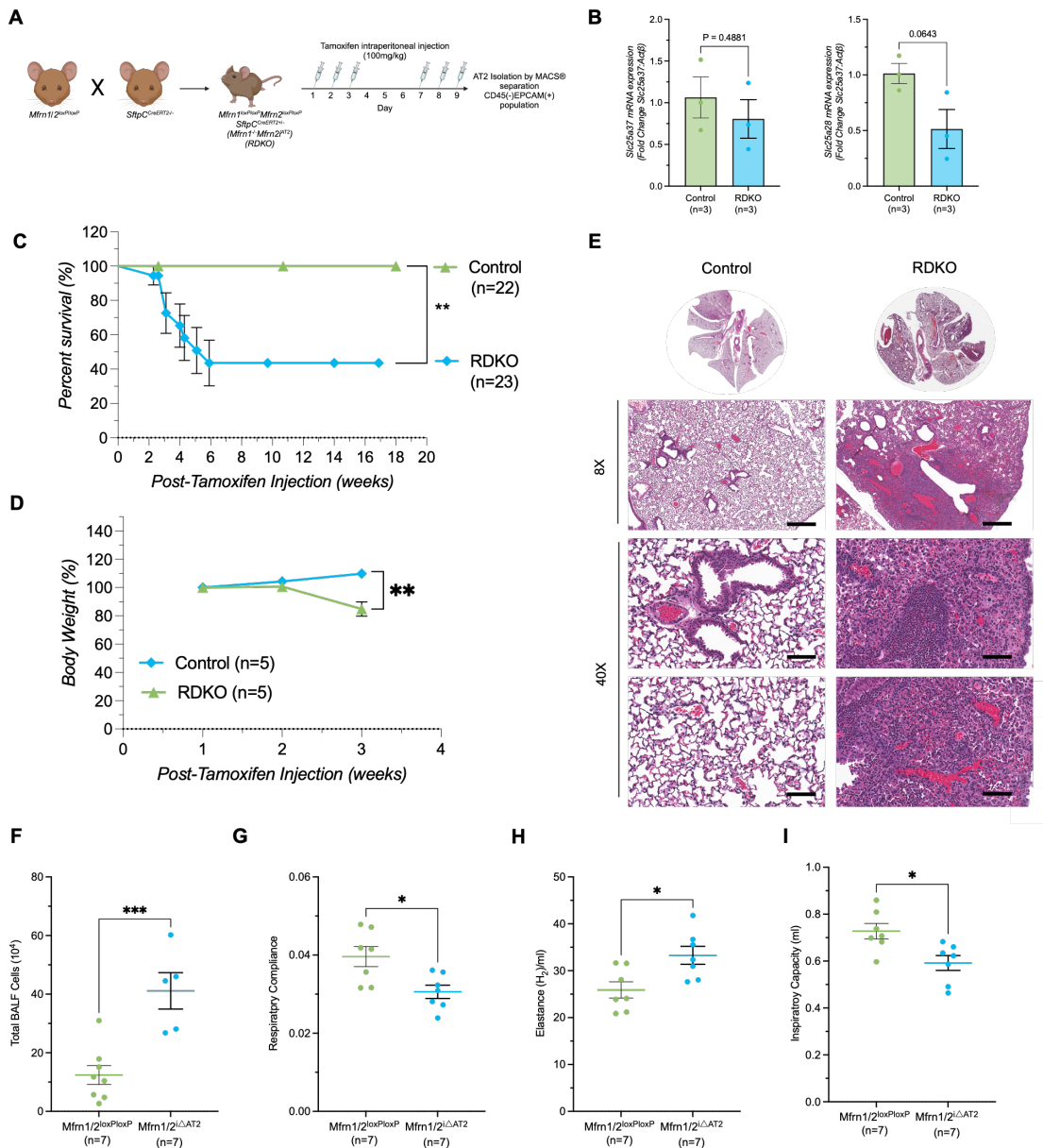


Figure 5.4: Homozygous loss of both *Mfrn1* and *Mfrn2* in AT2 cells leads to neutrophilic and proliferative interstitial pneumonia resulting in significant morbidity and mortality

(A) Schematic illustrating the generation of *Mfrn1^{loxP/loxP}Mfrn2^{loxP/loxP}Sftpc^{CreERT2}/+* (RDKO) mice, described in Chapter 2. Induction of the Lox-Cre system was achieved via six intraperitoneal tamoxifen injections administered over two weeks. (B) Gene expression analysis of *Mfrn1* (left) and *Mfrn2* (right) in isolated AT2 cells from RDKO mice. (C) Kaplan-Meier survival curve showing the percentage survival of RDKO mice compared to control mice after cessation of tamoxifen injections. (D) Percentage change in body weight over time, calculated for each mouse relative to its initial weight. (E) Haematoxylin and eosin staining of lung slices from control and RDKO mice. Objective Lens 8X and 40X. Scale Bar 50μM. (F) Total cell count (×10⁴) retrieved during the first 2.1 mL of BALF collection. (g-i) Lung function tests, including (G) respiratory compliance, (H) elastance, and (I) inspiratory capacity, measured in control and RDKO mice. Data are presented as mean ± SEM, with the number of biological replicates (n) indicated in each panel. Statistical significance was determined using an unpaired Student's t-test, with **p < 0.01, ***p < 0.001, and ****p < 0.0001. For body weight and survival curves, comparisons were made at specific time points between groups.

5.2.2.1 Refining the tamoxifen-inducible Cre/loxP system

To validate the previous model, we refined the method in accordance with local regulations in Ireland to comply with the Health Products Regulatory Authority (HPRA) 3Rs (Reduce, Refine, Replace). Previously, six intraperitoneal (IP) injections of TMX at 100 mg/kg over two weeks were used to activate the Cre system in AT2 cells (**Figure 5.4A**). In this project, we optimized the protocol by administering five subcutaneous (SC) TMX injections (100 mg/kg) over two weeks, followed by four weeks of TMX diet supplementation (**Figure 5.5A**). Gene expression analysis of isolated AT2 cells showed a significant reduction in *Mfn2* expression but not *Mfn1* (**Figure 5.5B**), with similar efficacy as the previous model (**Figure 5.4B**).

5.2.2.2 Exploring the efficacy of a refined tamoxifen-inducible Cre/loxP protocol.

As mentioned, previously three weeks after the IP injection protocol, RDKO mice lost about 30% of their body weight, a 50-60% mortality rate, with nearly all subjects dying 12 weeks after the cessation of TMX injections (**Figure 5.4C and 5.4D**). Under the refined SC and diet intervention, RDKO mice lost about 40% of their body weight three weeks after the last SC TMX injection, similar to previous results (**Figure 5.5C**). In accordance with HPRA welfare checks, mice were scored every three days after the cessation of TMX injections. Survival rates were not recorded in the refined method, as animals were sacrificed at humane endpoints, however, if an individual mouse reached a severity score of 5 or more, it was removed from the experiment and humanely euthanized. Notably, RDKO mice showed a rapid increase in severity scores when compared to the control group (**Figure 5.5D**). Control and RDKO mice injected with vehicle control (chen-oil) gained weight normally and showed minimal injection-related effects (**Figure 5.5C and 5.5D**). Thus, VC control groups were excluded from future experiments to reduce animal use.

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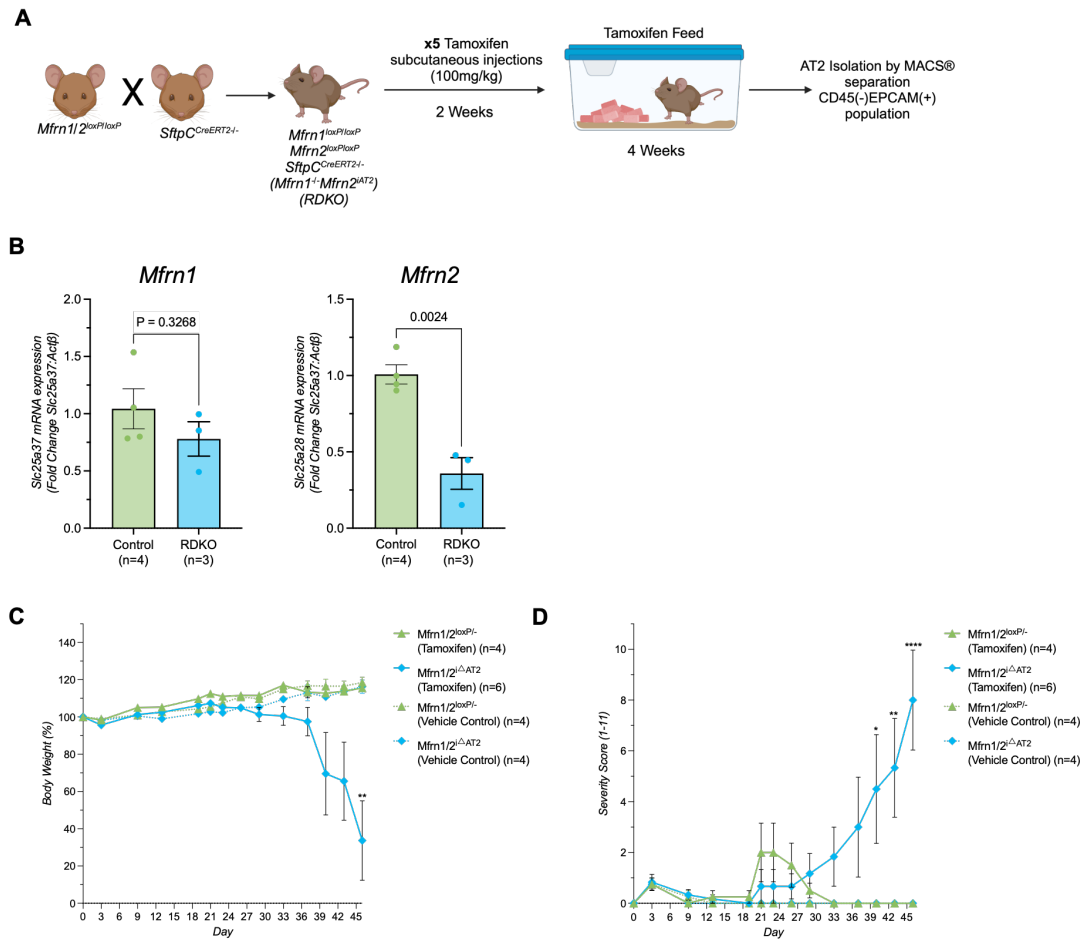


Figure 5.5: Subcutaneous injections and a tamoxifen-supplemented diet are sufficient to activate the lox/Cre system in RDKO mice

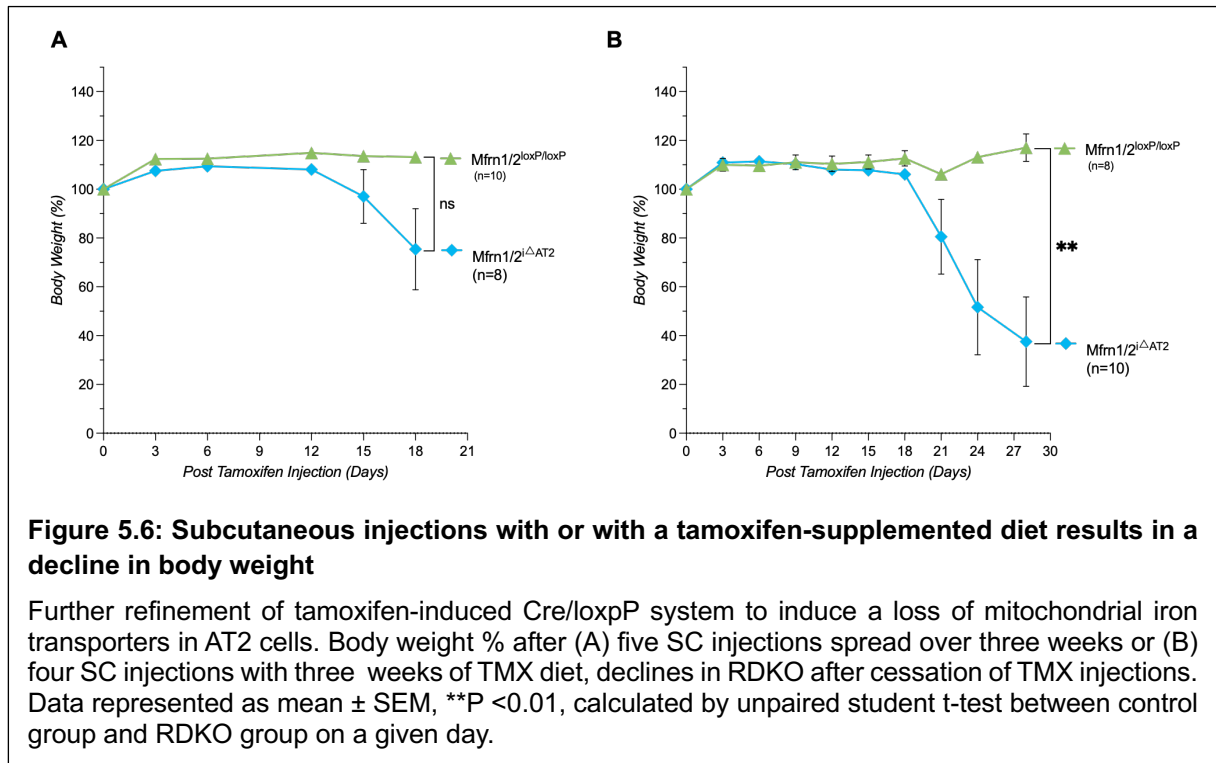
(A) Visual schematic of the refined tamoxifen procedure via the use of subcutaneous injections and diet intervention in *Mfrn1*^{loxP/loxP} *Mfrn2*^{loxP/loxP} *SfpC*^{CreERT2+/-} (RDKO) mice. (B) Gene expression analysis of *Mfrn1* (left) and *Mfrn2* (right) in isolated AT2 cells from RDKO mice compared to control mice (*Mfrn1/2*^{loxP/loxP}). Each individual data point represents an individual mouse. (C) Body Weight (%) of Control and RDKO mice given either tamoxifen or the vehicle control (VC), chen-oil. (D) Severity score of RDKO and control mice during SC injection and diet tamoxifen experimental procedure. Data represented as mean ± SEM, ##### P<0.0001, ## P<0.01, # P<0.05 ns = non-significant, by two-way ANOVA with Tukey's correction. *P <0.05, calculated by unpaired student t-test between control group and RDKO group.

5.2.2.3 Further refinement in the number of injections and diet administrated

As described, five SC injections and four weeks of TMX diet was efficient in recapitulating the previously observed results of six IP injections. We wanted to investigate whether or not this method could be refined further and employed two separate experiments where mice were either subjected to five SC injections spread over three weeks or four SC injections spread over two weeks followed by three weeks of diet. Similarly, both RDKO in the SC injections only group and the four SC injections and diet groups began to gradually lose weight 2-3 weeks

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after tamoxifen injection cessation (**Figure 5.6A and 5.6B**). The SC injection-only group exhibited a more progressive weight loss compared to the other groups (**Figure 5.6A**). Due to an increase in severity score, approximately 50% of the RDKO mice required humane euthanasia, resulting in no further body weight data being collected beyond three weeks in the SC injection-only group. Taken together, body weight analysis suggests successful activation of the Cre/loxP system across all methods. Subsequent experiments employed the most humane protocol: four subcutaneous injections over two weeks, followed by a two-week dietary regimen.



5.2.3 A Loss of Mitochondrial Iron Transport in AT2 Cells Drives AT2 Cell Dysfunction and Surfactant Dyshomeostasis

After recapitulating the RDKO model, we wanted to apply our AT2 functional readouts and investigate how a loss of mitochondrial iron transport in AT2 cells disrupts their function directly. Looking at AT2 related genes including surfactant related genes (*SftpC*, *Cd36* and *Fasn*) we observed a significant downregulation in all these markers (**Figure 5.7A**). Additionally, *Fgfr2*, a crucial signalling pathway for alveolar maintenance and AT2 cell fate (73), was also significantly downregulated when compared to controls (**Figure 5.7A**). Analysis of lung tissue and BALF revealed a loss of Pro-SFTPC protein in lung tissue and increased secretion of mSFTPC in BALF isolates (**Figures 7B and 7C**). Furthermore, BALF from RDKO mice exhibited significantly reduced surface tension levels when compared to controls (**Figure 5.7D**).

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Given the observed reduction in AT2 cell markers at both transcriptional and protein levels, we aimed to determine whether this could be attributed to an enrichment of AT1 cells within the isolated CD45⁺ EPCAM⁺ population. However, the expression levels of AT1-specific markers, Podoplanin (*Pdpn*) and Aquaporin5 (*Aqp5*), did not show an increase in the RDKO isolates (**Figure 5.7E**). Notably, there was a trend toward elevated transcription of the Keratin 8 (*Krt8*) marker, though the experiment lacked sufficient power to confirm statistical significance (**Figure 5.7E**). The upregulation of transitional cell markers, without a corresponding increase in AT1 markers, indicate that AT2 cells isolated from RDKO mice may be arrested in a transitional state rather than fully transdifferentiating into AT1 cells for epithelial repair.

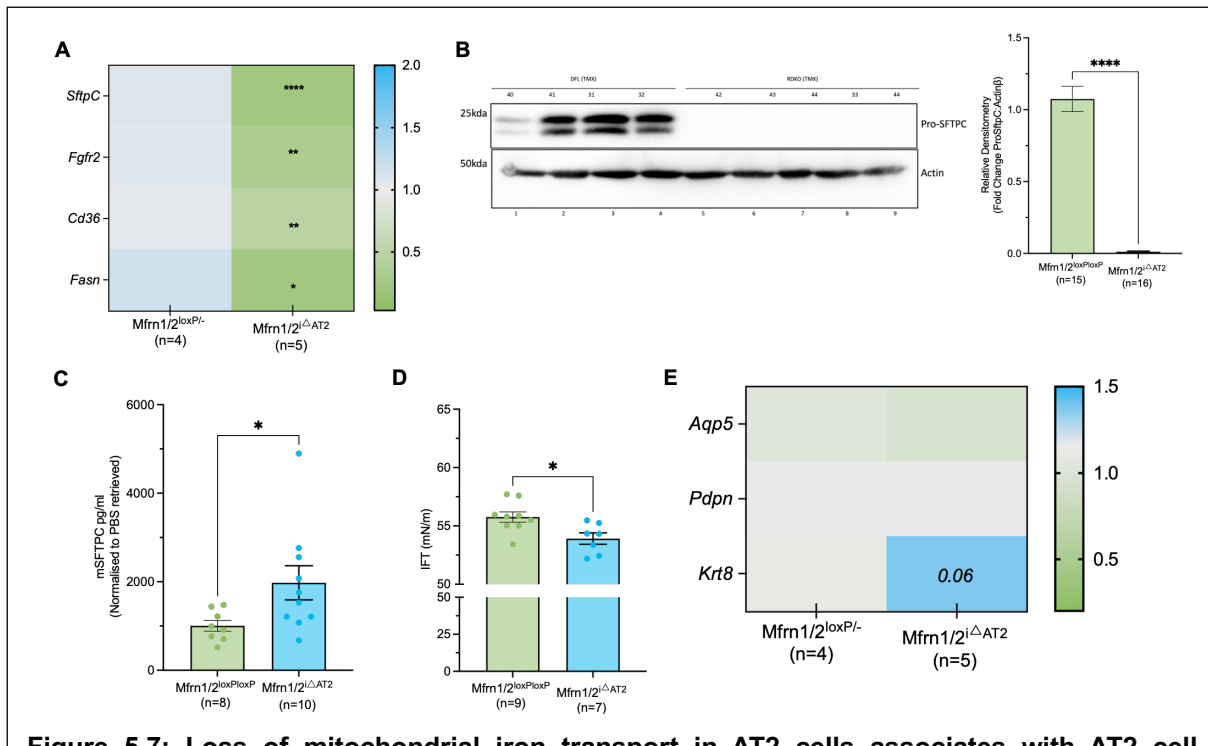


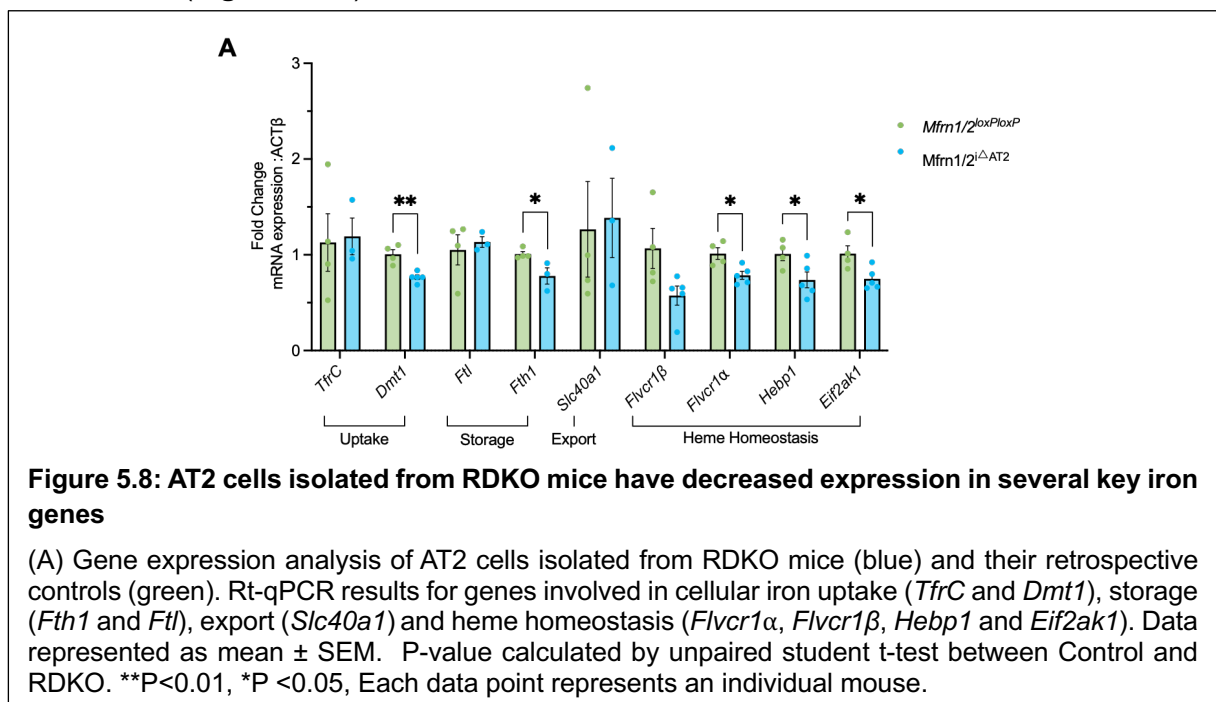
Figure 5.7: Loss of mitochondrial iron transport in AT2 cells associates with AT2 cell dysfunction, disrupting surfactant homeostasis

(A) Heatmap depicting gene expression analysis data represented as fold-change of AT2 cells isolated from RDKO mice and their retrospective controls. AT2 cell markers include; *Sftpc*, *Fgfr2*, *Cd36* and *Fasn*. (B) Representative immunoblot (left) with corresponding densitometry (right) for Pro-SFTPC in lung tissue from RDKO mice and control mice, with β -ACTIN serving as a control. (C) Secreted mSftPC in the BALF of RDKO (blue) and Control (green) mice. ELISA data (pg/mL) from each individual mouse was normalized to the volume of PBS recovered during lavage. (D) Biophysical properties of murine BALF in RDKO and control mice. Utilizing a sessile drop tensiometer, surface tension (IFT mN/m) of the fluid was calculated. (E) RTqPCR data represented as fold change of AT1 cell marker (*Pdpn* and *Aqp5*) and transitional cell marker (*Krt8*) in AT2 cells from RDKO and control mice. All data represented as mean $\pm$ SEM, unless stated otherwise. P-value calculated by unpaired student t-test between Control and RDKO. **** P<0.0001, **P <0.01, *P <0.05, n number depicted in each panel.

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5.2.3.1 Reduced intracellular iron in AT2 cells disrupts lung iron homeostasis and drives tissue damage and inflammation

Isolated AT2 cells from RDKO mice also exhibited a dysregulated iron transcriptome, characterized by a marketed downregulation of key iron regulatory genes, including those involved in iron import (*Dmt1*), storage (*Fth1*), and heme metabolism (*Flvcr1 α* , *Flvcr1 β* , *Hebp1*, *Eif2ak1*) (**Figure 5.8A**). Specifically, the *Dmt1* gene was significantly decreased, suggesting reduced NTBI uptake. The classic iron uptake (*Tfrc*) and export (*Slc40a1*) remained unchanged. Notably, the expression of *Fth1*, *Flvcr1 α* , and *Hebp1* were significantly decreased, with a trend toward decreased *Flvcr1 β* . This gene expression profile suggests that AT2 cells may be iron and heme-deficient, as they are downregulating storage (*Fth1*), export (*Flvcr1 α* and *Flvcr1 β*), and heme-binding (*Hebp1*) genes. In the HetDKO model, the loss of *Mfrn1* and *Mfrn2* results in reduced intracellular iron and heme levels in AT2 cells (**Figures 1D and 1E**), but did not induce upregulation of *Hri* expression. Similarly, RDKO-AT2 cells fail to exhibit compensatory upregulation of *Hri* despite pronounced alterations in iron and heme homeostasis (**Figure 5.8A**).



To explore the systemic implications of AT2-specific iron deficiency on pulmonary iron regulation, we next measured total iron levels in digested lung tissue using GF-AAS. This analysis revealed significantly reduced total iron levels in the lung (**Figure 5.9A**). Consistently, TFRC protein levels were significantly elevated, indicating a compensatory response to iron deficiency at the tissue level (**Figure 5.9B**). Paradoxically, the iron storage protein FTH1 was also upregulated, despite decreased total lung iron content (**Figure 5.9D**). HRI protein trended toward downregulation (**Figure 5.9D**), suggesting impaired iron-deficient sensing or failure of

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the HRI-ISR axis in the lung. These findings underscore how iron dysregulation in AT2 cells can propagate iron dyshomeostasis throughout lung tissue.

Further assessment of lung health revealed significant stress and inflammatory signatures.

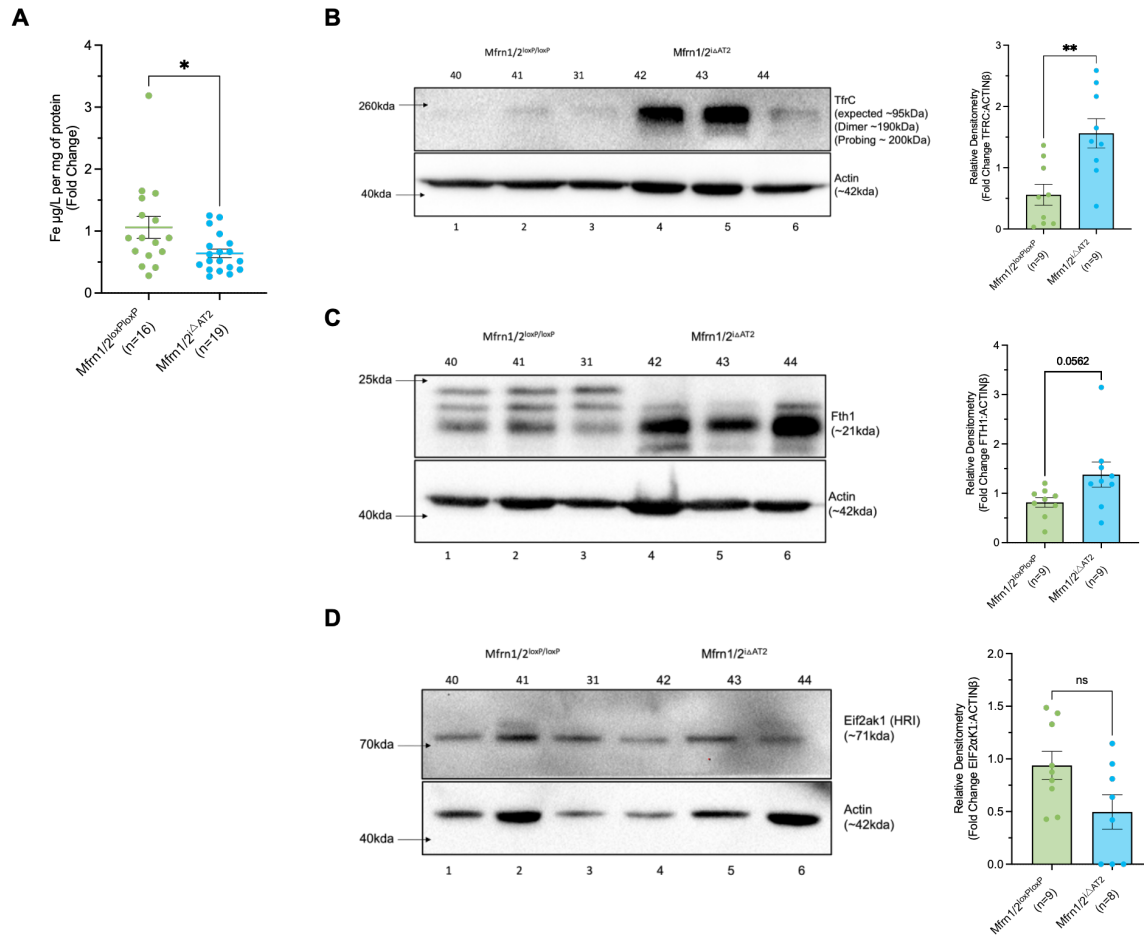


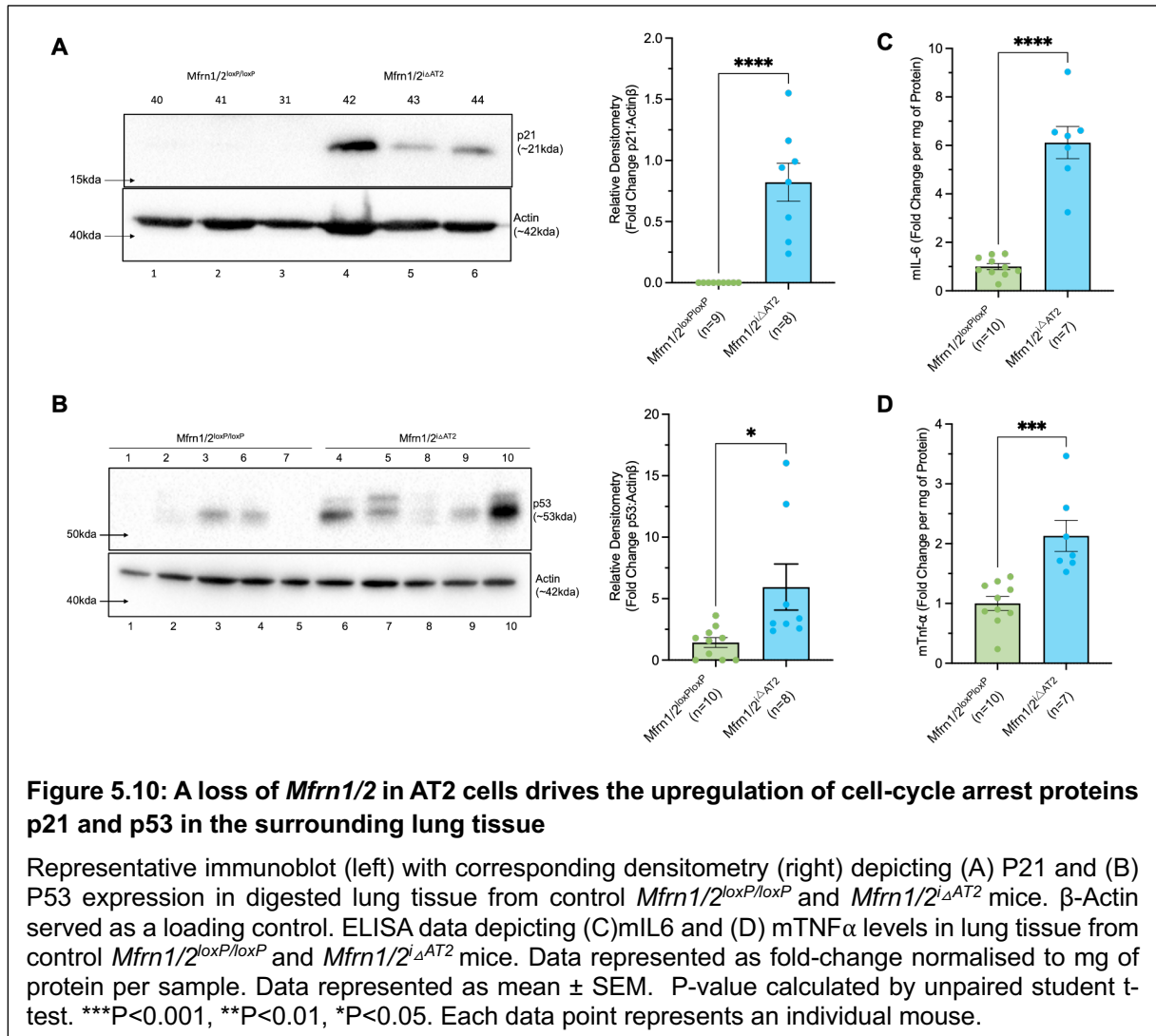
Figure 5.9: A loss of mitochondrial iron transport in AT2 cells disrupts lung iron homeostasis

(A) GF-AAS data depicting total lung iron levels in digested lung tissue from RDKO mice and their retrospective controls. Representative immunoblot (left) with corresponding densitometry (right) depicting (B) TfRc, (C) FTH1 and (D) EIF2AK1 (HRI) expression in digested lung tissue from control (*Mfrn1/2^{loxP/loxP}*) and RDKO (*Mfrn1/2^{ΔAT2}*) mice. β -Actin served as a loading control. Data represented as mean $\pm$ SEM. P-value calculated by unpaired student t-test. ** $P < 0.01$, * $P < 0.05$, with each data point representing an individual mouse.

Whole-lung tissue displayed elevated protein expression of p53 and its downstream effector p21 (**Figures 5.10A and 5.10B**), indicating widespread cell-cycle arrest and the potential for increased apoptosis among resident lung cells. Concurrently, there was a dramatic increase in pro-inflammatory cytokines IL6 and tumour necrosis factor TNF- α in the lung tissue (**Figures 5.10C and 5.10D**), suggesting a heightened inflammatory response. Collectively, these findings illustrate substantial tissue damage and inflammation in the lung, stemming from

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impaired MITOIRON transport in AT2 cells. This underscores the critical role of mitochondrial iron regulation not only in maintaining AT2 cell function but also in preserving overall lung homeostasis and preventing tissue injury.

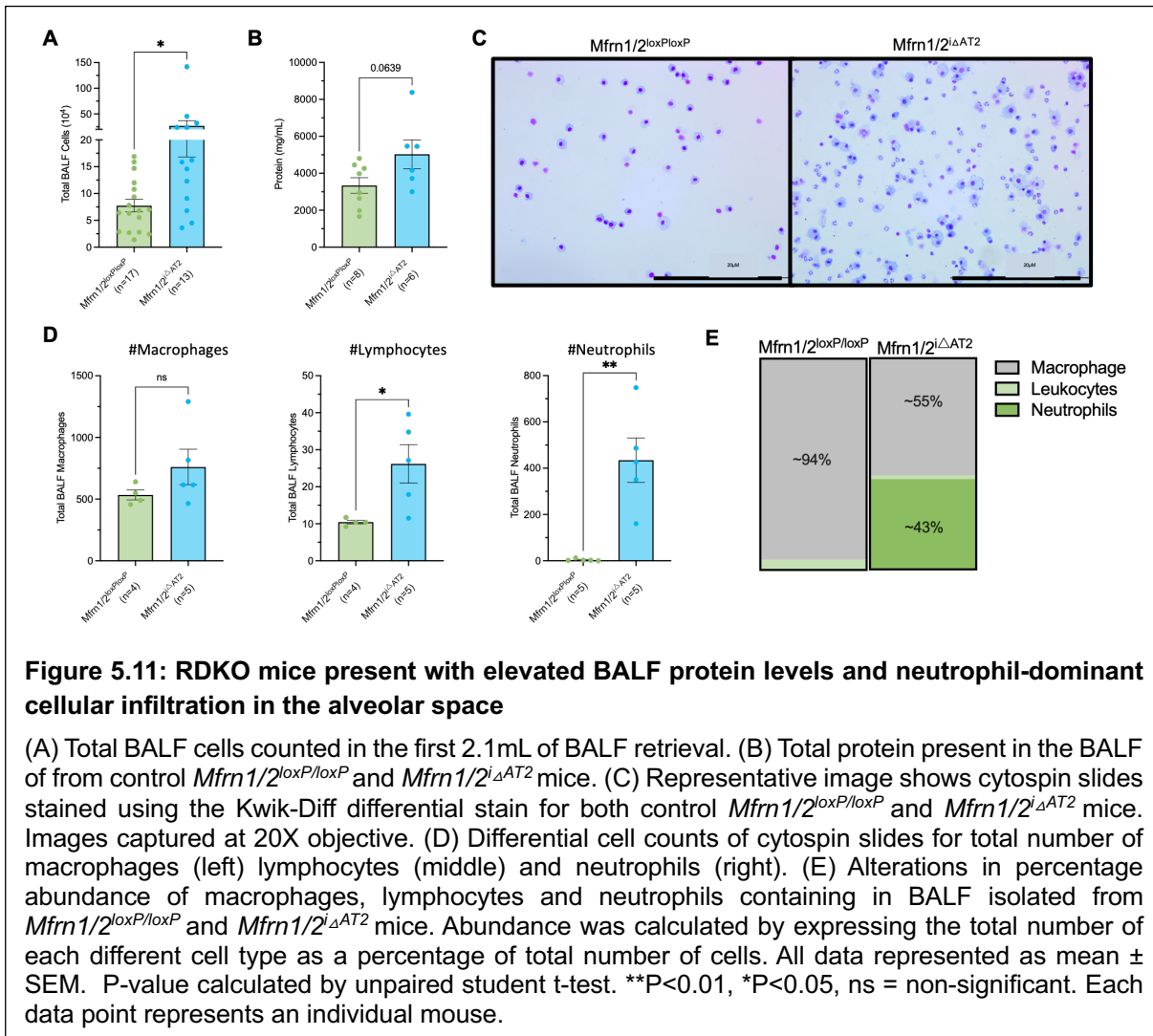


5.2.3.2 A loss of mitochondrial iron transport in AT2 cells increases BALF iron and drives cellular infiltration and inflammation

To further investigate the impact of iron-driven AT2 cell dysfunction, we assessed changes in the lung microenvironment via BALF. As previously shown (**Figure 5.4F**), RDKO mice exhibit a significant increase in the total number of cells in the BALF (**Figure 5.11A**). Additionally, RDKO mice showed a trend toward elevated protein levels in the BALF, which may suggest injury or increased permeability of the alveolar-capillary barrier.

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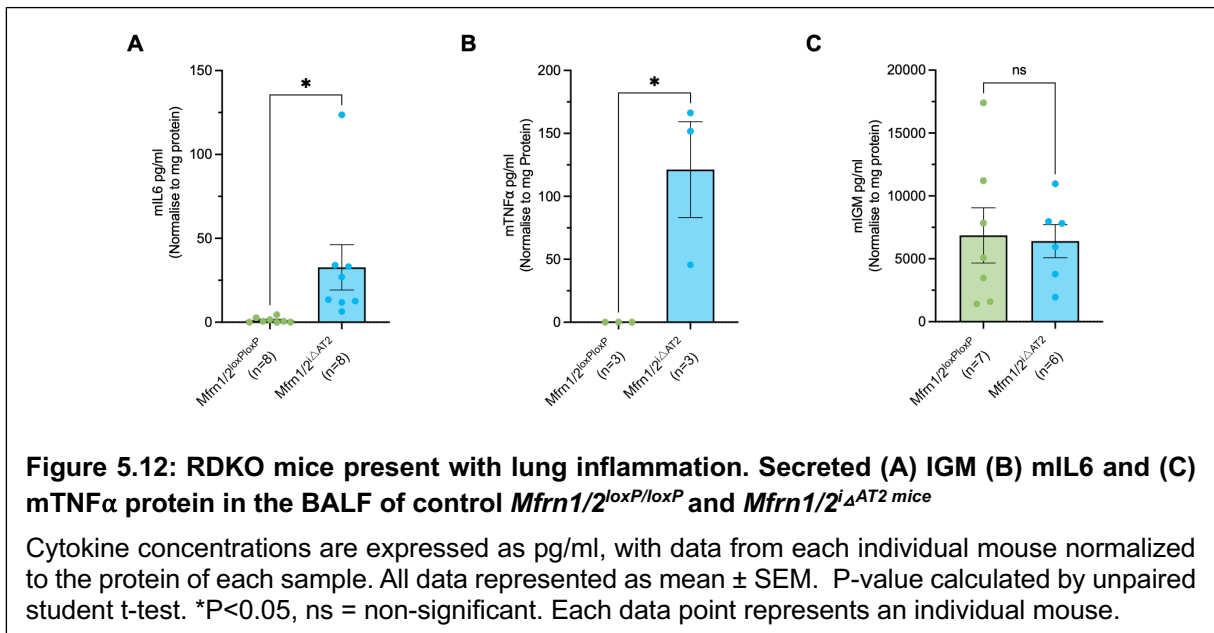
Using the refined method of *Mfrn1/2* deletion in AT2 cells *in vivo*, differential cytopsin analysis of BALF cells revealed an increase in infiltrating lymphocytes and neutrophils (**Figures 5.11C and 5.11D**). Strikingly, more than 50% of immune cells in the BALF of RDKO mice were neutrophils, resulting in a disproportionate neutrophil-to-macrophage ratio. In contrast, macrophages accounted for ~90% of resident BALF immune cells in controls (**Figure 5.11E**). These findings suggest that while macrophages remain present, they are vastly outnumbered by neutrophils, positioning neutrophils as the primary drivers of immune cell infiltration in this model.



Consistent with the increase in immune cell populations, RDKO mice also displayed significantly elevated levels of pro-inflammatory cytokines $\text{TNF}\alpha$ and IL-6 in the BALF (**Figures 5.12A and 5.12B**). Despite increased cellular infiltration and BALF protein, IGM, a serum derived protein and marker of barrier integrity in the lung remained unchanged in the BALF

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(Figure 5.12C). Collectively, these findings highlight the profound impact of iron-driven AT2 cell dysfunction on lung immune cell infiltration, inflammation, and damage in the alveolar space.



In contrast to a loss of iron in the lung tissue of RDKO mice, loss of *Mfrn1/2* in AT2 cells significantly increased total iron levels in the BALF (Figure 5.13A). To account for potential bias from elevated protein and cellular infiltration (Figure 5.11A), total iron was normalized to protein levels, revealing consistently elevated iron levels (Figure 5.13B). Elevated iron in BALF is a well-established biomarker of pulmonary injury and is implicated in the pathogenesis of various chronic lung diseases (182). Further investigations into iron accumulation in the BALF revealed significantly elevated concentrations of heme (Figure 5.13C). More detailed analysis of the heme constituents demonstrated a significant increase in protoporphyrin levels (Figure 5.13D).

To explore the relationship between BALF iron levels and immune cell infiltration, we analysed the correlation between iron concentrations (measured via GF-AAS) and the total number of infiltrating cells (Figure 5.13E). A moderate positive correlation was observed between the two variables, with further stratification by cell type revealing the strongest associations between BALF iron levels with neutrophil and macrophage counts (Figure 5.13D). Taken together, this suggests that neutrophils and macrophages may serve as a key source of the iron influx into the alveolar space. However, further investigations are needed to confirm this hypothesis and elucidate underlying mechanisms.

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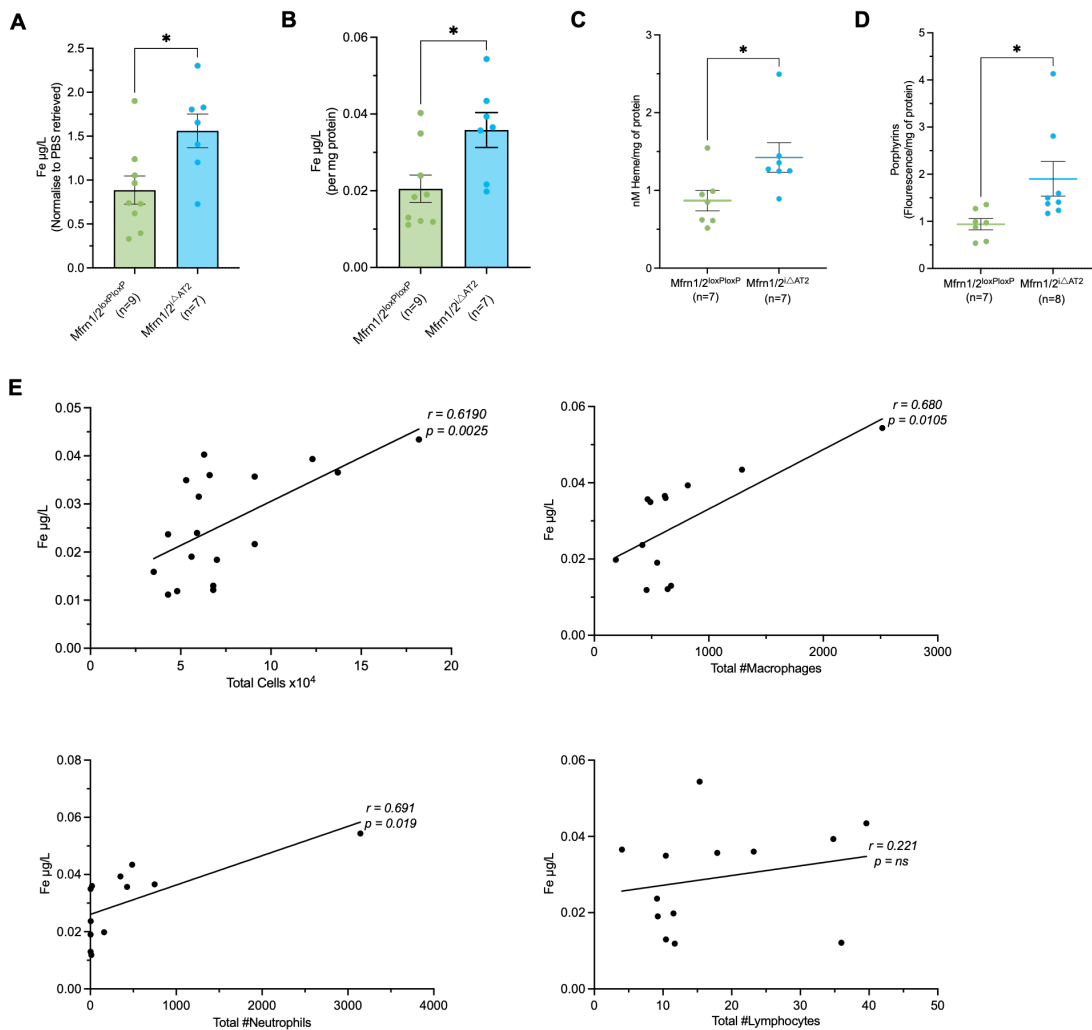


Figure 5.13: A loss of *Mfrn1/2* in AT2 cells drives iron dyshomeostasis in the alveolar microenvironment

Total iron (Fe $\mu\text{g/L}$) levels in the BALF of control *Mfrn1/2^{loxP/loxP}* and *Mfrn1/2^{ΔAT2}* mice (A) normalized to the volume of PBS recovered during lavage and (B) the amount of protein present in the BALF. Data represented as mean $\pm$ SEM. P-value calculated by unpaired student t-test, * P<0.05. (C) Total heme levels and (E) endogenous porphyrins (non-heme protoporphyrin IX and naturally occurring porphyrins) in BALF normalised to mg of protein in each sample from RDKO and control mice. Analysis of the correlation between BALF iron levels and total cell counts (top left), total macrophages (top right), total neutrophils (bottom left), and total lymphocytes (bottom right). The linear relationship between BALF iron levels and cell counts was quantified using the Pearson correlation coefficient (r). Statistical significance (p-value) was determined using a t-test, where r represents the correlation coefficient and n indicates the sample size. Each data point represents an individual mouse.

5.2.4 Establishing a Cross-Talk Experimental Model Between AT2 Cells and AMs *In Vitro*

Having identified macrophages as potential contributors to iron dysregulation in the BALF, we next sought to investigate how AT2 cells might influence the iron profile and function of AMs. To explore these mechanisms, we developed an *in vitro* "cross-talk" model to study the interactions between MLE-12 cells, a murine AT2-like cell line, and foetal liver-derived alveolar macrophages (FLAMs), a murine alveolar macrophage-like model (211). Both cell types are specialized and require specific growth media to maintain their phenotype. FLAMs, derived from foetal monocytes and cultured in GM-CSF and TGF- β , exhibit characteristics of alveolar macrophages, making them suitable for our studies (211). MLE-12 cells are cultured in HITES medium, modified with transferrin (10 μ g/ml), 2% FBS, and HEPES (10 mM), to preserve AT2-like characteristics (244).

To identify a compatible growth medium for both cell types, we supplemented HITES medium with GM-CSF and TGF- β (referred to as HITES++). Alamar Blue assays confirmed that HITES++ supported the growth and metabolic activity of both MLE-12 cells and FLAMs, with the latter showing increased metabolic activity (**Figure 5.14A**). To avoid challenges from

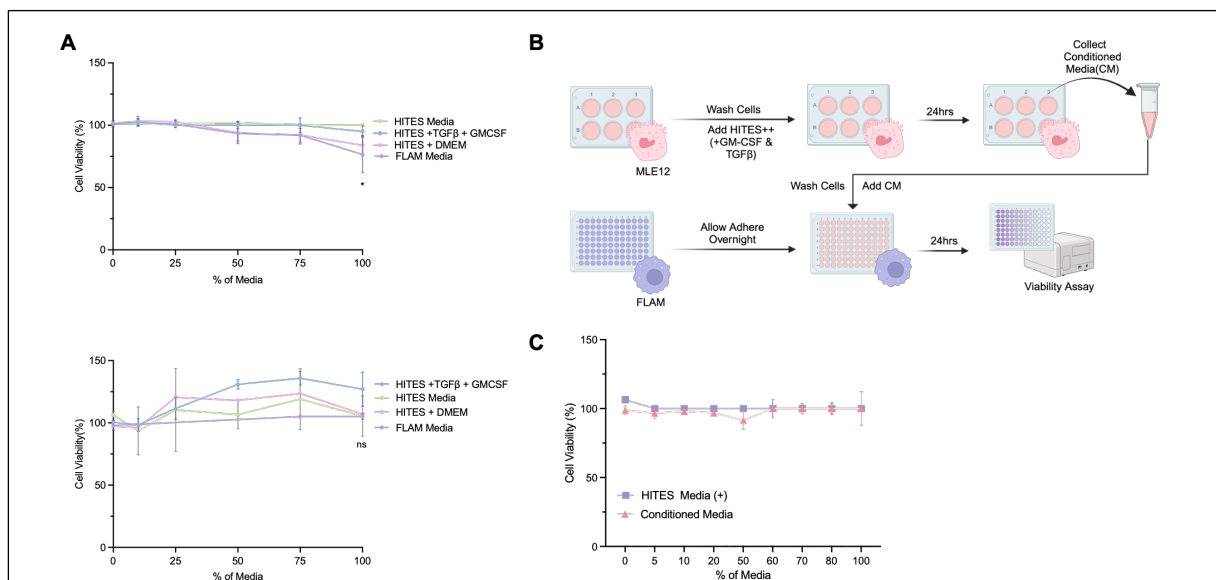


Figure 5.14: Establishing a cross-talk experimental model between MLE12 cells and FLAM cells

(A) Cellular viability in MLE12 cells (top) and FLAM cells (bottom) when treated with HITES media, HITES supplemented medium (+TGF β and +GMCSF), HITES media + DMEM and FLAM media for 24hrs. P-value calculated by unpaired student t-test comparing FLAM media to HITES media control, * P<0.05. (n=3 independent biological replicates). (B) Graphical illustration of conditioned media experiments. (C) Cellular viability of FLAM cells incubated with conditioned medium from MLE12 cells, with HITES supplemented medium serving as the untreated control. All data represented as mean $\pm$ SEM. (n=3 independent biological replicates).

isolating single-cell suspensions for downstream analysis in co-culture conditions, we utilized

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a conditioned media (CM) approach to assess the effects of MLE12-secreted factors on FLAMs (**Figure 5.14B**). MLE-12 cells were plated and allowed to adhere overnight, then washed and incubated in HITES++ for 24 hours to collect secreted factors. The CM, after centrifugation to remove debris, was applied to previously plated FLAM cells. Results showed that MLE-12 cell derived CM had no significant effect on FLAM viability, as measured by Alamar Blue (**Figure 5.14C**).

We first assessed how MLE12 cell-derived CM affected FLAM function (**Figure 5.15A**). Analysis of key FLAM markers (surfactant lipid uptake (*Cd36*, *Pprγ*), pathogen clearance (*Marco*, *Cd11c*), and resolution of inflammation (*Pprγ*) showed no significant transcriptional changes in response to MLE12-CM (**Figure 5.15B**). However, CM exposure significantly reduced expression of iron-regulatory genes involved in import (*Tfrc*), export (*Slc40a1*), and storage (*Fth1*) (**Figure 5.15C**). Suggesting the secretome from a healthy MLE-12 cell may dampen iron metabolism in FLAM cells.

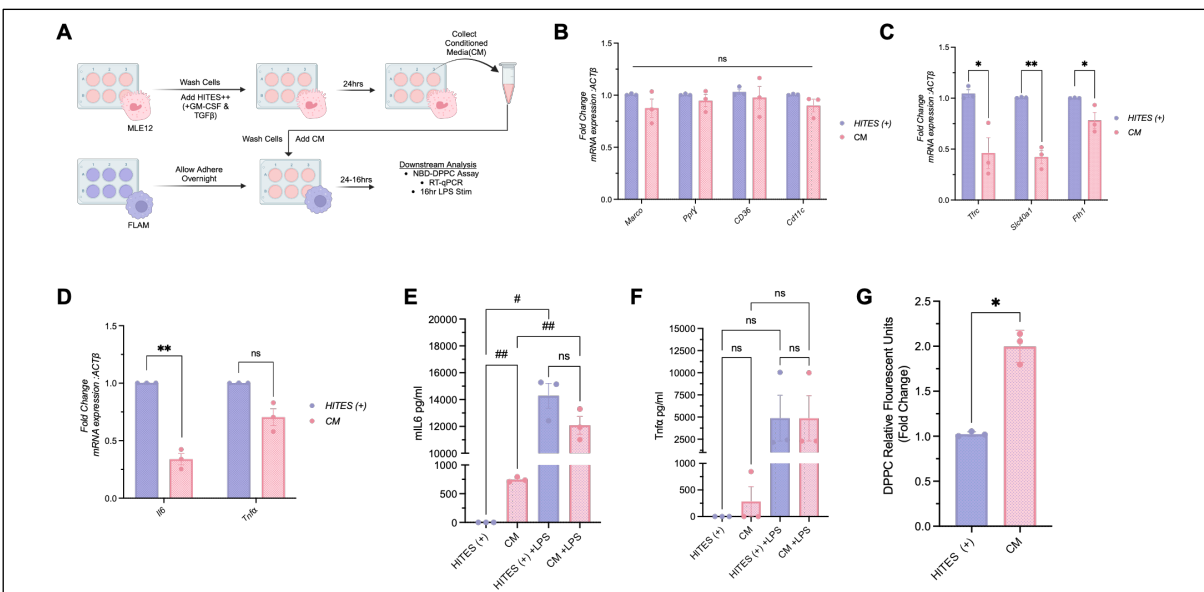


Figure 5.15: Conditioned media from MLE12 cells attenuates FLAM cell immune response to LPS and enhances surfactant lipid uptake capacity

(A) Graphical summary of conditioned media experiments. For LPS stim, FLAM cells were incubated in conditioned media for 16 hours, washed, and stimulated with 100 ng LPS overnight. Media and cells were collected for RT-qPCR analysis. For NBD-DPPC uptake, FLAM cells were treated with conditioned media for 24 hours, washed, and subjected to the uptake assay. Gene expression in FLAM cells for (B) alveolar macrophage functional markers: *Cd36*, *Marco*, *Pprγ* and *Cd11c*. (C) cellular iron metabolism genes: storage (*Fth1*), uptake (*Tfrc*), and export (*Slc40a1*); and (D) pro-inflammatory cytokines: *Il6* and *Tnfα*. ELISA data for secreted (E) mIl6 and (F) mTnfα from FLAM cells incubated in conditioned media from MLE12 cells, and subsequently stimulated with 100ng of LPS compared to their retrospective controls. (G) NBD-DPPC uptake assay in FLAM cells with or without conditioned media from MLE12 cells. All data represented as mean ± SEM. P-value calculated by either unpaired student t-test (*) or one-way Anova (#) with Tukey's correction. P < 0.01, #/*P < 0.05, ns = non-significant. Each data point represents an independent biological replicate.

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Given the central role of AMs in pulmonary immune defence, we examined how MLE-12 cell CM influenced FLAM immune responses upon LPS stimulation. In the lung, AT2 cells are known to regulate AM inflammation by maintaining a dampened alveolar microenvironment to prevent excessive immune activation (40, 41). Consistent with this, FLAMs pre-incubated with MLE12-CM showed reduced transcriptional expression of pro-inflammatory cytokines *Il6* and *Tnfa*, with a statistically significant reduction in *Il6* levels (**Figure 5.15D**). FLAMs stimulated with LPS secreted more IL6 and TNF α (**Figure 5.15E and 5.15F**). Notably, FLAMs pre-treated with MLE-12 CM prior to LPS stimulation showed a trended reduction in *IL6* secretion, while *TNF α* levels remained unchanged (**Figure 5.15E and 5.15F**).

Lastly, as AMs play a critical role in surfactant turnover (296), we assessed FLAM surfactant uptake using a previously established NBD-DPPC uptake assay (**Chapter 3**). FLAMs exposed to MLE-12 cell derived CM for 24 hours demonstrated significantly increased uptake of NBD-DPPC, indicating enhanced surfactant clearance (**Figure 5.15G**). Taken together, these results demonstrate the significant influence of AT2 cells on AMs, shaping their iron metabolism, inflammatory responses, and capacity to uptake surfactant lipids.

5.2.4.1 FAC and DFX supplementation in MLE12 cells associates with dysregulated iron metabolism in FLAMs

After establishing an *in vitro* cross-talk model, we next sought to determine how altered iron levels in MLE12 cells affect FLAM function. Using previously established iron-replete (FAC) and iron-deplete (DFX) models (**Chapter 4**), MLE-12 cells were treated with 100 μ M FAC or 10nM DFX for 24 hours. Following treatment, cells were washed and incubated in HITES++ medium for an additional 24 hours to collect CM (**Figure 5.16A**).

FLAMs exposed to FAC- or DFX-treated MLE-12 cell CM showed no significant changes in the expression of AM genes (**Figure 5.16B**). However, both FAC-CM and DFX-CM increased the expression of iron export (*Slc40a1*) and iron uptake (*Tfrc*) genes in FLAMs, while DFX-CM also reduced the expression of the iron storage gene (*Fth1*). Although these findings are underpowered for definitive conclusions, the increased expression of both *Tfrc* and *Slc40a1*, would suggest dysregulated iron metabolism in FLAMs and a loss of the IRP-IRE axis.

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Next, we evaluated NBD-DPPC uptake in FLAMs in response to iron loaded or iron deficient MLE-12 cells. FAC-CM significantly impaired surfactant uptake, while DFX-CM had no effect (**Figure 5.16C**). Consistent with these findings, iron-loaded MLE-12 cells similarly reduced DPPC uptake (**Chapter 4**). Together, these results suggest that elevated iron levels impede lipid uptake in both FLAMs and MLE-12 cells, indicating a potential mechanistic link between iron overload and disrupted surfactant lipid uptake.

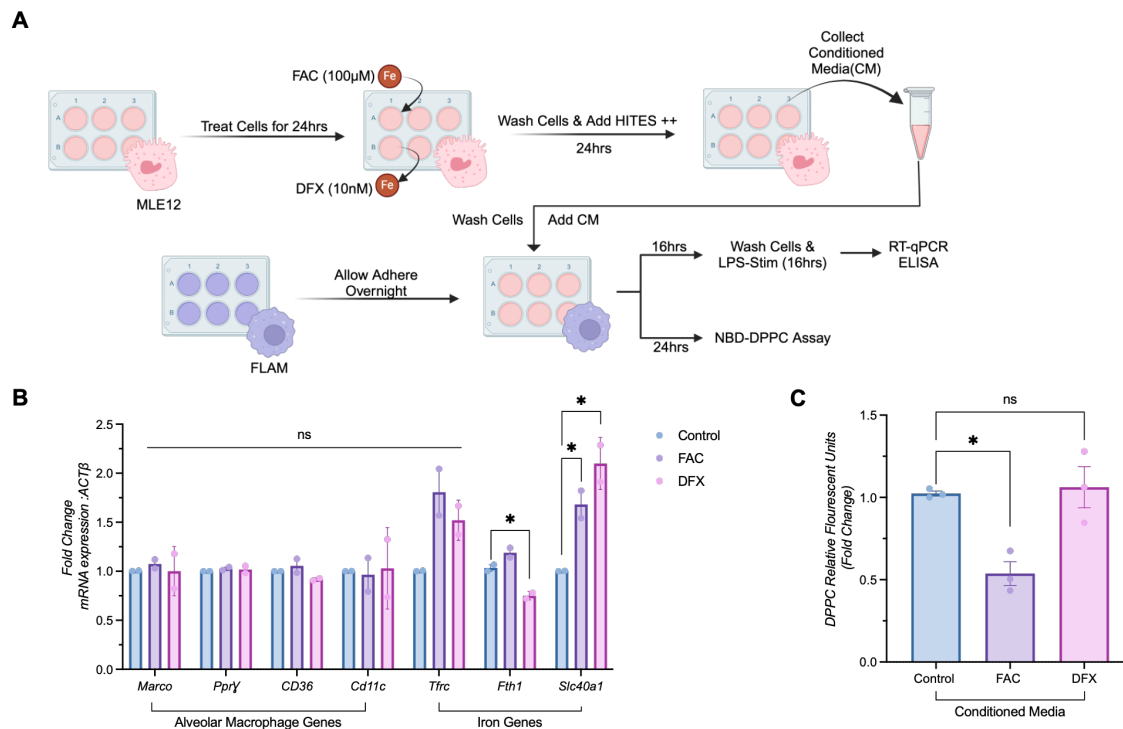


Figure 5.16: Conditioned media from iron-replete MLE12 cells decrease surfactant lipid uptake in FLAM cells

(A) Graphical summary of conditioned media experiments. (B) Gene expression in FLAM cells for alveolar macrophage functional markers and cellular iron metabolism genes normalized to β -actin as the housekeeping gene and represented as fold change. (C) NBD-DPPC uptake assay in FLAM cells treated with conditioned media from MLE12 cells. All data represented as mean $\pm$ SEM. P-value calculated by unpaired student t-test, *P < 0.05, ns = non-significant. Each data point represents an independent biological replicate.

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Lastly, we assessed the inflammatory response of FLAMs treated with FAC-CM, DFX-CM, or Control-CM (**Figure 5.17A**). Consistently, *Il6* transcription also trended toward higher levels with DFX-CM treatment, with an amplified response observed after LPS stimulation, suggesting a pro-inflammatory effect of DFX-CM on FLAMs (**Figure 5.17A**). LPS stimulation did not significantly alter the expression of iron-related genes in FLAMs, regardless of CM condition (**Figure 5.17B**). In summary, altered iron levels in AT2 cells modulates FLAM iron metabolism, impairs surfactant uptake, and influences inflammation. Iron-deplete conditions amplify FLAM inflammatory signalling, while iron-replete conditions disrupt lipid processing, highlighting the complex interplay between AT2 iron homeostasis and alveolar macrophage function.

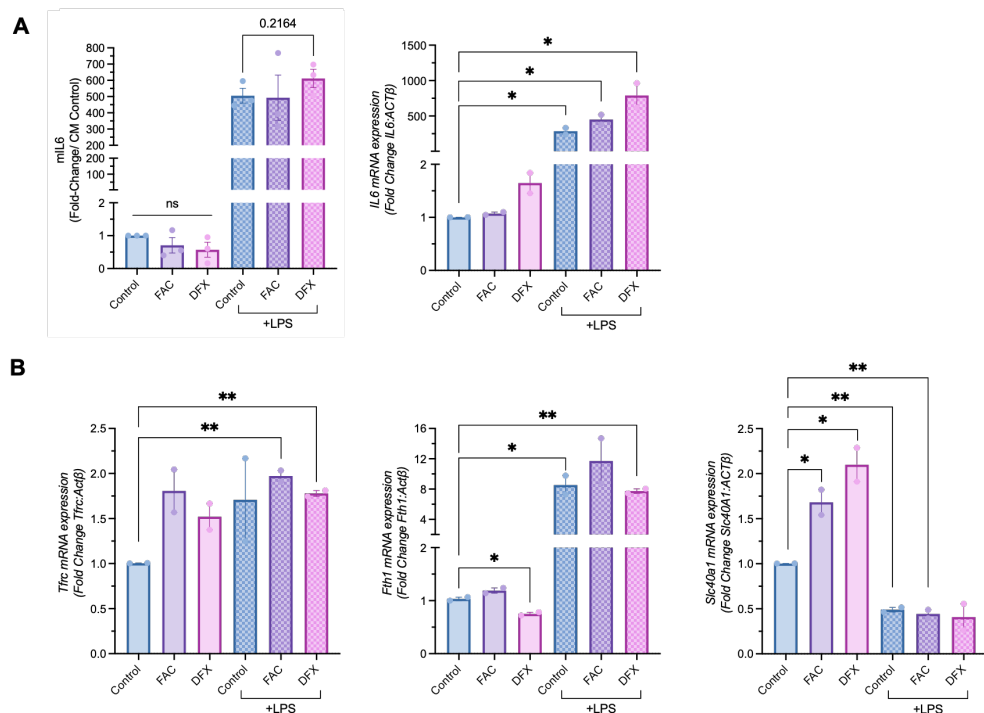


Figure 5.17: Conditioned media from iron deplete MLE12 cells exacerbate FLAM cells inflammatory response to LPS

(A) Secreted protein (left) and gene expression (right) values for *Il6* in FLAM cells treated with conditioned media from iron-replete (FAC), iron-deplete (DFX) and control (Water) MLE12 cells and subsequently stimulated with 100ng of LPS for 16hrs. (B) RT-qPCR gene expression in FLAM cells for *Tfrc* (left), *Fth* (middle) and *Slc40a1* (right). All data represented as mean $\pm$ SEM. P-value calculated by unpaired student t-test **P < 0.01, *P < 0.05, ns = non-significant. Each data point represents an independent biological replicate.

5.3 Discussion

Throughout this thesis, the significance of AT2 cells and the molecular mechanisms underlying their function has been a central focus. **Chapter 4** demonstrates that proper iron handling is crucial for AT2 functions, including surfactant homeostasis and self-renewal *in vitro*. In this Chapter findings were extended, revealing that mitoferrin 1 and 2 are essential for AT2 cell function and lung health *in vivo*. Disruption of MITOIRON transport in AT2 cells leads to cellular and lung iron imbalances, causing AT2 dysfunction, severe alveolar neutrophilia, acute lung injury, and ultimately mortality. Building on the cellular dysfunction induced by AT2-specific iron dysregulation, we demonstrate that alveolar macrophages are similarly affected *in vitro*. Disruptions in MLE12 cell iron homeostasis alter FLAM cell iron metabolism, impair surfactant uptake, and modulate inflammatory responses. These findings underscore the pivotal role of iron regulation in AT2 cells for maintaining lung homeostasis and highlight its potential as a therapeutic target to prevent or mitigate lung disease progression.

5.3.1 Iron Regulation in AT2 Cells *In Vivo*.

Consistent with our *in vitro* findings and previous literature, loss of *Mfrn1* and *Mfrn2* specifically in AT2 cells significantly reduces their total cellular iron and heme levels (149-152, 156). In response to intracellular iron depletion, AT2 cells in HetDKO mice appear to upregulate iron uptake and transport pathways, likely compensating for decreased intracellular iron. In contrast, AT2 cells from RDKO mice exhibited decreased expression of iron uptake and storage genes, suggesting that the IRP-IRE iron signalling system may be impaired. This discrepancy likely stems from the severe damage and functional decline observed in RDKO AT2 cells, reflected by reduced expression of AT2-specific markers.

Additionally transcriptional alterations to the Ironome include significant increased expression in several Fe-S and heme synthesis genes, suggesting that *Mfrn1/2* HetDKO AT2 cells may be attempting to enhance mitochondrial metabolism given the reduced availability of iron, heme, and Fe-S clusters. Additionally, HO-1 expression is increased. Given the reduced heme levels in these cells, it is likely that HO-1 is upregulated for its anti-inflammatory and antioxidant properties (297). Additionally *Prdx1* and *Slc25a39*, both involved in mitigating oxidative stress (294, 295), were significantly increased, suggesting that disruption of MITOIRON transport may impair oxidative homeostasis.

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In HetDKO mice, heterozygous loss of *Mfrn1* does not significantly impair the animals health. This could be due to haplo-sufficiency, where one functional *Mfrn1* allele maintains sufficient gene expression (298). Interestingly, the *Abcb10* gene, which complexes with MFRN1 to regulate mitochondrial iron transport in erythroblasts (152), is significantly increased in this model, suggesting transport may still be facilitated. In yeast, loss of both *Mrs3* and *Mrs4* (*Mfrn1/2* homologs) decreases mitochondrial iron levels under iron-limiting conditions, indicating that other low-affinity mitochondrial iron transporters may operate when iron levels are in abundance (149, 150). In this model, the upregulation of *Mcu*, which has been shown to facilitate ferrous iron transport into the mitochondrion under specific conditions, may compensate for mitoferrin deficiency (299).

Another potential compensatory mechanism involves the upregulation of *Slc25a39*, a transporter implicated in iron incorporation into protoporphyrin IX and the mitochondrial import of glutathione (GSH) (295, 300, 301). GSH, an essential cytosolic antioxidant, and mitochondrial iron are tightly linked, with MFRNs stabilizing SLC25A39 to maintain a balanced GSH-to-iron ratio (295, 300). Consistently, recent studies showed that the loss of either *MFRN1* or *MFRN2* alone permits SLC25A39 stabilization under GSH-deficient conditions (300). Whereas dual loss of both mitoferrins disrupts this stabilization, indicating that a single functional mitoferrin may be sufficient to stabilize SLC25A39.

Additionally, SLC25A39 stabilization is influenced by Fe-S cluster biogenesis (295, 300). AT2 cells from HetDKO mice showed increased expression of several Fe-S biogenesis genes. Together, disruption of both mitochondrial iron transport and Fe-S biogenesis would likely impair SLC25A39 stabilization, although the remaining functional *Mfrn1* allele in HetDKO mice may allow partial stabilization and transport. Future studies into SLC25A39 protein levels, particularly in AT2 cells from RDKO mice, where mitoferrin transport is severely disrupted would provide insight into whether or not mitochondrial iron can be transported via SLC25A39 when both mitoferrins are lost.

As discussed, both MFRNs have been linked to heme metabolism (149-152, 156). In agreement, AT2 cells from HetDKO mice have significantly reduced intracellular heme levels. Heme levels in AT2 cells from RDKO was not assessed, however, given the strong correlation between a loss of either *Mfrn1* or *Mfrn2* and a reduction in heme levels in both the literature and in **Chapter 4** we assume the same is true for RDKO AT2 cells. RNAseq data from HetDKO AT2 cells showed downregulation of the mitochondrial heme exporter *Pgmc2* (essential for signalling heme transport to the nucleus) (163), upregulation of *Slc25a39* (involved in iron incorporation into protoporphyrin IX) (301), upregulation of the heme importer *Slc48a1* (HRG1)

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[3], and loss of *Alad* (an enzyme in the porphyrin and heme biosynthesis pathway) (302). In agreement, AT2 cells from RDKO also present with decreased expression in heme exporters *Flvc1ra* and *Flvcr1b*.

AT2 cells from HetDKO and RDKO mice exhibit significantly reduced expression of the *Hri* gene, aligning with the *in vitro* findings in MLE12 cells deficient in *Mfrn1* or *Mfrn2* (**Chapter 4**). As part of the ISR, HRI is activated under iron and heme depletion (303). In erythrocytes, heme binding modulates HRI's activity by inhibiting its kinase function, balancing globin synthesis with available heme (304). Despite decreased intracellular iron and heme levels, AT2 cells do not upregulate HRI expression. These observations suggest that *Mfrn1* and *Mfrn2* may influence the ISR-HRI pathway in specialized AT2 cells, highlighting their potential regulatory roles in cellular stress responses. Of note, the ISR-HRI axis remains inactive in RDKO lung tissue despite reduced total iron levels. HRI protein expression is decreased in digested lung tissue, potentially driven by AT2 cells, as suggested by previous findings. Alternatively, elevated iron and heme in BALF may mask local iron deficiencies, preventing ISR-HRI activation.

5.3.2 The Effect of Impaired MITOIRON Transport on AT2 Cell Function.

In agreement with our *in vitro* findings, AT2 cells from HetDKO and RDKO mice have dysregulated surfactant homeostasis. Specifically, AT2 cells from HetDKO mice upregulate several ATP synthase subunits (305), including *Atp5a1*, *Atp5b*, *Atp5g3*, and *Atp5o*. In addition, genes such as *Aco2*, *Aco1*, *Sdhb*, *Cox7a2*, *Surf1*, and multiple complex I subunits are significantly upregulated. Given that mitochondrial metabolism is highly iron-dependent (167), with many of these genes encoding enzymes requiring iron–sulfur clusters or heme as cofactors, this transcriptional shift likely reflects a compensatory response to impaired ETC function and reduced ATP production following the loss of mitoferrin 1/2. This is consistent with the observed downregulation of key mitochondrial genes involved in energy metabolism, such as *Pdp2* (a regulator of the pyruvate dehydrogenase complex), *Dnaj15* (a co-chaperon involved in oxidative phosphorylation and lipid metabolism) *Ndufs6* (complex-I sub-unit) and *Dld* (a mitochondrial enzyme).

Regarding surfactant proteins, transcripts for *SftpB* and *SftpC* are significantly increased in AT2 cells from HetDKO mice. Conversely, *SftpC* expression in AT2 cells, along with Pro-SFTPC protein levels in lung tissue, are significantly decreased in RDKO mice. This

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discrepancy likely reflects the extent of AT2 cell damage and the loss of AT2 cell markers in RDKO mice. Intriguingly, gene expression analysis of AT2 cells isolated from RDKO mice reveals a potential enrichment of cells expressing the transitional cell marker *Krt8* (75), which may explain the reduced *SftpC* expression. The complete absence of Pro-SFTPC in lung tissue may be due to interference from the Cre system linked to the SFTPC promoter, which could affect antibody binding and warrants further investigation. Notably, the presence of mSFTPC in the BALF indicates that protein synthesis, processing and secretion is still occurring. Interestingly, RDKO mice exhibit increased mSFTPC levels in their BALF, which may suggest enhanced secretion, as mSFTPC is typically packaged into lamellar bodies and secreted alongside surfactant lipids (3).

Despite increased mSFTPC levels, the BALF exhibits reduced maximum surface tension capacity, suggesting impaired surfactant secretion from AT2 cells. Together this suggests that the observed increase in mSFTPC could result from defective turnover or degradation of surfactant. Impaired surfactant turnover and uptake in AT2 cells lacking mitochondrial iron transporters is further supported by findings from **Chapter 4**. Specifically, *Mfrn1*^{CRISPRi} and *Mfrn2*^{CRISPRi} cells exhibit decreased surfactant lipid uptake. Furthermore, AT2 cells from HetDKO mice show significantly reduced expression of *Adgrf5* (adhesion G-protein coupled receptor F5), a critical regulator of surfactant clearance and turnover. Loss of *Adgrf5* in mice results in abnormal surfactant accumulation and airway inflammation, with AT2 cell-specific deletion of *Adgrf5* recapitulating this phenotype, whereas endothelial-specific deletion does not (306, 307). Taken together, while direct comparisons to SFTPC protein turnover cannot be made, it is plausible to hypothesize that AT2 cells from RDKO mice, similar to AT2 cells in HetDKO mice and *Mfrn1/2*^{CRISPRi} cells, have impaired surfactant turnover and uptake capacity.

AT2 cells were not isolated to directly assess regenerative or transdifferentiation capacity in HetDKO and RDKO mice. However, in RDKO mice, the extent of lung damage, increased mortality, loss of AT2/AT1 cell markers, and enrichment of *Krt8* gene expression suggest that AT2 cells lose their repair capacity when mitochondrial iron transport is impaired. Furthermore, HetDKO mice exhibit disruptions in the expression of several NADH:Ubiquinone Oxidoreductase Subunit (*Ndufs*) genes, which encode Fe-S cluster-containing proteins essential for complex-I, a critical component of the mitochondrial respiratory chain (167). Recently, it was shown that disruptions to complex-I activates stress response pathways in AT2 cells, which prevents the successful differentiation into AT1 cells (70). In our model, loss of mitochondrial iron transport disrupts iron metabolism, impairing Fe-S biogenesis and Fe-S dependent proteins which are essential for complex-I function. While the central role of complex-I dysfunction in this model remains to be determined, the consistent pattern of

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mitochondrial-related dysfunction upon loss of *Mfrn1* and *Mfrn2*, both *in vitro* and *in vivo*, may suggest these transporters play a role in AT2 cell stem-cell characteristics, warranting further investigation.

An additional pathway affected by a loss of mitochondrial iron transport in AT2 cells is the FGF signalling pathway, which plays a critical role in epithelial proliferation, repair, and surfactant synthesis (308, 309). *Fgf7* is among the most downregulated genes in AT2 cells from HetDKO mice. Although FGF7 is primarily secreted by fibroblasts, it is taken up by AT2 cells via FGFR2, whose transcript levels are significantly reduced in RDKO AT2 cells. Interestingly, *in vitro* loss of either *Mfrn1* or *Mfrn2* increases *Fgfr2* expression, suggesting that the presence of at least one mitoferrin is sufficient to sustain this pathway. Notably, *Fgf7* has been identified as a susceptibility locus for COPD, where the risk allele reduces FGF7 secretion in fibroblasts (310). Furthermore, exogenous FGF7 protects against BLM-induced fibrosis by suppressing TGF β expression (311). Together, these findings underscore the critical role of FGF signalling in AT2 cell repair and regeneration. Here, we propose a direct link between impaired MITOIRON transport and negative regulation of FGFR2 signalling, potentially contributing to the loss of AT2 cell repair capacity in RDKO mice.

5.3.3 The Impact of AT2 Cell-Specific MITOIRON Dysregulation on Lung Health.

To date, no studies have directly linked AT2-specific iron dysregulation to a decline in lung health. Here, we provide novel evidence that AT2-specific MITOIRON deficiency significantly impacts lung health by driving alveolar neutrophilia, inflammation, and iron dyshomeostasis in the alveolar space leading to acute lung injury and mortality.

As discussed, surfactant clearance/uptake is decreased in AT2 cells with disrupted MITOIRON transport. AT2 cells from HetDKO mice exhibit downregulation of the GM-CSF receptor, *Csf2ra*, which is crucial for AT2 cell function (312). GM-CSF is essential for stimulating surfactant catabolism in AMs, with disrupted GM-CSF signalling impairing AM surfactant clearance, leading to protein and phospholipid accumulation in the alveoli (313). *In vitro*, from iron loaded MLE-12 cells significantly decreases surfactant lipid uptake in FLAM cells. Given the significant increase in BALF iron levels, reduced surfactant clearance, and impaired communication with AMs, further investigation is warranted to understand how these factors affect AMs' ability to effectively catabolize surfactant *in vivo*.

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Disruptions in AT2 cell function can exacerbate alveolar inflammation. In health, AT2 cells modulate resident immune cells through secretory functions preventing inappropriate inflammation (40, 41). The immunomodulatory effect of AT2 cells is compromised by depleted intracellular iron levels *in vitro* and *in vivo*. Previous research demonstrated that silencing airway hepcidin induced AM iron deficiency, exacerbating lung injury responses and diminishing AM phagocytic capacity (194). This highlights the critical role of airway-derived iron proteins in modulating resident cells iron metabolism and immune function.

Here we show a loss of mitochondrial iron transport in AT2 cells drives severe immune cell infiltration and inflammation in the alveolar space. Previously, pro-inflammatory secretomes from lung epithelial cells were shown to activate AMs, which subsequently triggers neutrophil infiltration (40, 41, 314). Additionally, heme and its by-products activate neutrophils and facilitate their chemotaxis (315). Elevated BALF heme in RDKO mice likely results from haemorrhage and hemoglobin influx rather than AT2 cells. However, the lack of significant IGM changes suggests that this increase may not be solely due to vascular permeability disruption and may involve local lung cells contributions.

Limited research links heme and porphyrin accumulation to lung diseases, though sustained protoporphyrin IX (PPIX) buildup induces oxidative stress and lipid peroxidation in the liver, underscoring its pathological potential (316). PPIX accumulation is influenced by iron availability and FECH activity, as seen in fibroblasts from protoporphyria patients (317). Of note, MFRN1 deficiency is associated with IRP1-mediated inhibition of ALAS2 translation, preventing excessive PPIX accumulation (318). However, further IRP1 loss disrupts this regulatory feedback, exacerbating PPIX buildup (318). As discussed previously, AT2 cells from RDKO mice shown signs of disruptions in the IRP-IRE axis, which is further mirrored in the lung tissue. RNAseq from HetDKO AT2 cells suggests that porphyrin biosynthesis remains active, with a trend toward increased *Cpox*, *Ppox*, and *Fech* expression (**Figure 5.18**), suggesting that iron insertion is the rate-limiting step. Collectively, these findings suggest that *Mfrn1/2* loss in AT2 cells reduces intracellular iron and heme levels, impairing IRP-IRE regulation and potentially driving porphyrin accumulation, positioning AT2 cells as a key source of porphyrins in the BALF. Conversely, the heme/porphyrin exporter *Flvcr1a* is significantly downregulated in AT2 cells from RDKO mice. Evidence from other cell types suggests additional porphyrin export mechanisms (319, 320), which may similarly facilitate AT2-mediated porphyrin export into the BALF.

Expanding on porphyrin accumulation, AT2 cells from HetDKO mice significantly increase *Hebp1*. HEBP1 is integral to the synthesis of F2L, a high-affinity ligand for formyl peptide

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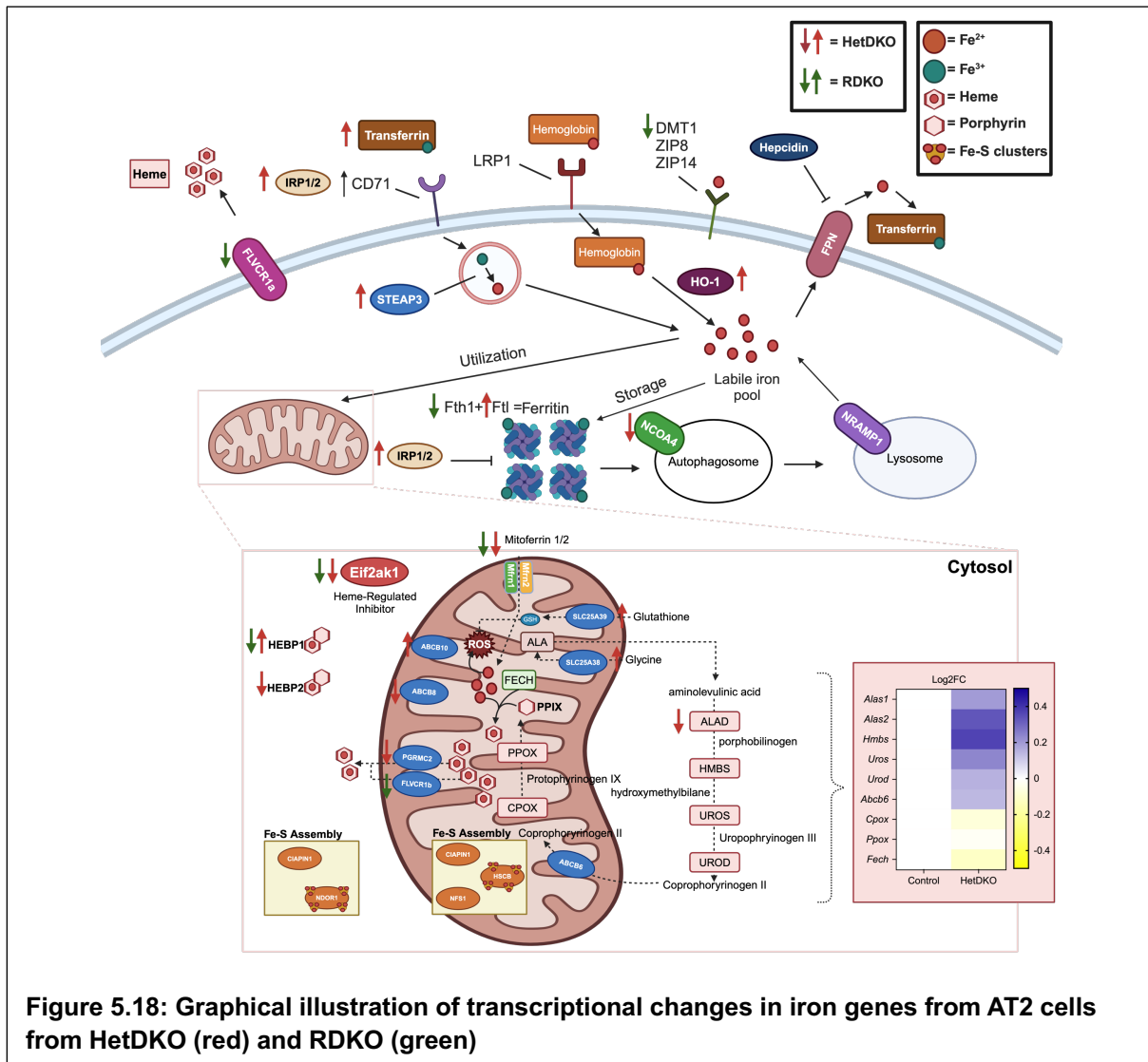


Figure 5.18: Graphical illustration of transcriptional changes in iron genes from AT2 cells from HetDKO (red) and RDKO (green)

receptor (FPR) 3 (321), which is expressed on monocytes, myeloid, and plasmacytoid dendritic cells in the lung (322). Interactions between F2L and FPR3, or its murine counterpart FPR2, stimulate neutrophil migration (323). Furthermore, recent studies highlight FPR signalling as a key contributor to lung inflammation, particularly in neutrophilic pneumonia and lung injury models (324). Conversely, AT2 cells from RDKO mice exhibit significantly reduced expression of the F2L precursor, *Hebp1*. This reduction could be attributed to the timing of cell isolation, post-establishment of neutrophilic infiltration and inflammation, indicating a possible loss of this signalling pathway due to the extent of damage and ongoing inflammation. Whereas, the HetDKO model appears to reflect the acute or earlier stages of dysfunction. Together, these findings suggest that impaired mitochondrial iron transport in AT2 cells may initiate HEBP1-FPR signalling, contributing to neutrophil infiltration and lung inflammation.

5.4 Limitations

While our findings provide novel insights into the essential role of MITOIRON transport in AT2 cell function and lung homeostasis, several limitations should be considered when interpreting our results.

The TMX-inducible Cre/loxP system, widely used for conditional gene deletion, exhibits variability in recombination efficiency and tissue specificity (325). Differences in TMX administration (e.g., injection vs. dietary supplementation) may impact recombination rates and gene expression. TMX is an estrogen receptor antagonist, which can induce off-target effects like interfering with hormone signalling (325). Although no sex-specific differences emerged in our readouts, potential estrogen-dependent effects cannot be excluded and warrant investigation in future studies. To control for TMX specific side effects, tamoxifen was administered to control animals. While no standard control exists, most studies use a mix of Cre- and Cre+ Flox- controls. Here, in the comparative medicine unit in Trinity College Dublin, only Cre- (*Mfrn*^{1/2^{loxP/loxP}) controls were available due to breeding constraints. Given that Cre systems can exhibit leakiness and off-target effects, it would have been preferable to include both Cre-negative and Cre-positive Flox-negative controls; therefore, the use of only Cre-negative controls represents a limitation of this thesis.}

An additional limitation of the current study is the lack of mechanistic insight into inflammatory pathways at play or further iron parameters disrupted in this model. Our results indicate that AT2-specific iron deficiency promotes inflammation, immune cell infiltration, and alveolar damage, yet the precise molecular mechanisms driving these effects remain unclear. While we observed increased pro-inflammatory cytokines (e.g., IL-6, TNF- α) and immune cell recruitment, additional mechanistic studies are needed to determine whether these changes are a direct consequence of AT2 cell stress, a secondary response to iron accumulation in the alveolar space, or a result of dysregulated local immune cell function.

During the assessment of iron parameters in lung tissue, this study focused only on total iron and protein readouts while excluding heme, non-heme iron and whole lung analysis with BALF. The elevated TFRC and FTH expression suggests potential dysregulation of the IRP-IRE signalling pathway, as these proteins are typically inversely regulated. Despite these molecular changes, total iron levels in digested lung tissue were significantly reduced, likely due to prior BALF isolation, which includes multiple PBS washes that may remove iron associated with

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haemorrhage or tissue damage. Consistently, RDKO mice exhibited significantly higher iron and heme levels in BALF. This highlights the need for a multifaceted approach to look at all angles i.e. all forms of iron to get a bigger picture of the iron-status in the lung.

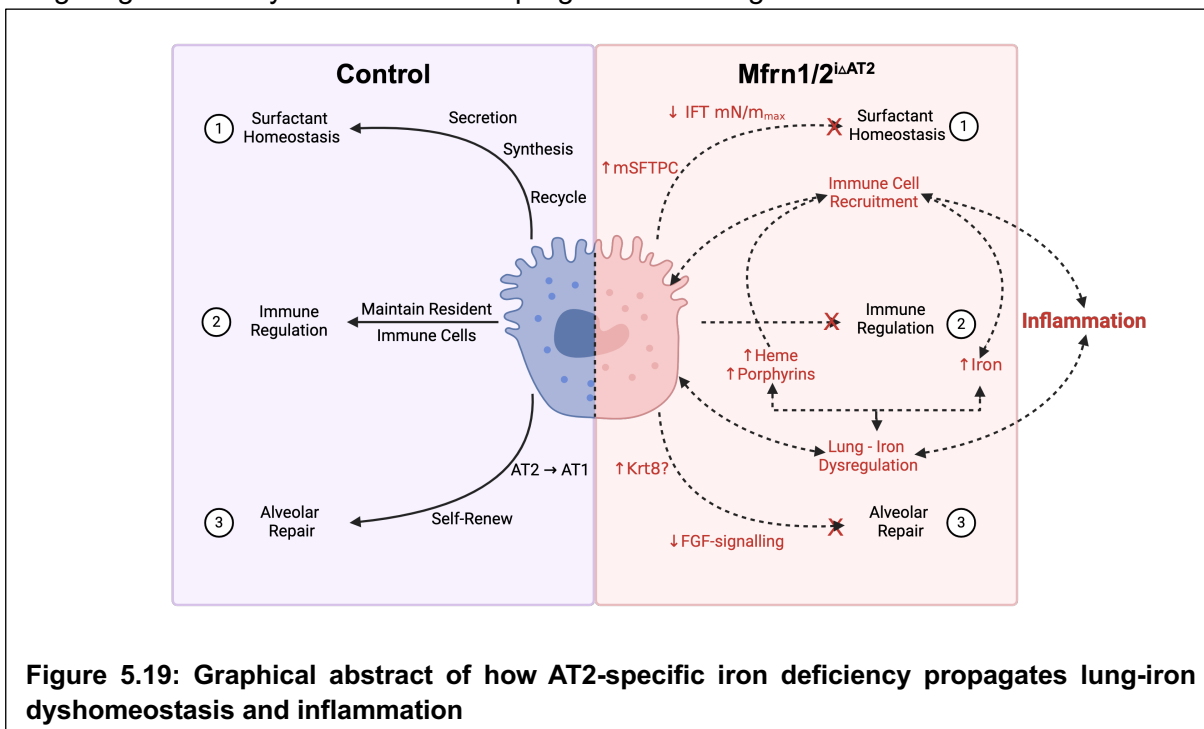
In vitro cross-talk model between MLE-12 cells (AT2-like) and FLAMs (AM-like) provided valuable insights into AT2-AM interactions but has inherent limitations. MLE-12 cells, while widely used, do not fully replicate the phenotype and metabolic characteristics of primary AT2 cells. Similarly, FLAMs may not completely mimic resident alveolar macrophages in an adult lung environment. The use of primary murine AT2 cells and AMs would provide a more physiologically relevant system for future studies. Additionally, direct cell to cell communication is lost in the CM model.

5.5 Conclusions

This chapter provides the first evidence that mitoferrin 1 and 2 are indispensable for AT2 cell function and overall lung health. Through a combination of *in vitro* and *in vivo* approaches, we establish that mitochondrial iron transport via *Mfrn1/2* is critical for AT2 cell-specific functions, including surfactant homeostasis, metabolic regulation, and epithelial repair by sustaining intracellular iron and heme levels.

AT2-specific iron deficiency lead to secondary iron dysregulation in the lung, resulting in the accumulation of iron, heme, and porphyrins within the alveolar space. Concurrently, *Mfrn1/2* deficiency in AT2 cells promotes immune cell infiltration, exacerbating inflammation and cellular stress, ultimately culminating in acute lung injury. We propose that persistent inflammation and impaired alveolar repair due to AT2-specific iron deficiency establishes a self-perpetuating cycle, in which lung-iron dysregulation and inflammatory signalling sustains and amplifies tissue damage, ultimately leading to injury and mortality (**Figure 5.19**).

Our findings redefine the role of AT2 cells in lung disease, demonstrating that AT2-specific iron homeostasis is fundamental not only for AT2 cell viability but also for orchestrating immune responses, regulating surfactant metabolism, and facilitating tissue repair. By understanding the mechanistic links between AT2-specific iron dysfunction and pulmonary pathology, our results highlight AT2-MITOIRON regulation as a promising therapeutic target for preventing or mitigating AT2 cell dysfunction and the progression of lung disease.



6. Iron in AT2 Cell Injury and Dysfunction

6.1 Introduction

AT2 cells play a pivotal role in the repair and regeneration of the alveolar epithelium, often positioning them at the forefront of research into the pathogenesis of chronic lung diseases, including IPF and COPD. COPD is primarily characterized by chronic inflammation and emphysema, which culminates in significant airflow limitation (326). Conversely, IPF is marked by progressive irreversible fibrosis, leading to a reduction in lung volume (327). Despite the distinct different pathological features of these diseases, they share several age-related mechanisms, including mitochondrial dysfunction, telomere shortening, aberrant repair processes, and cellular senescence. Additionally, both IPF and COPD are associated with disrupted lung iron homeostasis, leading to iron accumulation in the lungs of affected patients (114, 115). Despite the established role of iron dysregulation in both diseases, its specific effects on AT2 cell dysfunction and senescence remains poorly understood, necessitating further investigation.

Cellular senescence represents a complex and dynamic cellular stress program, hypothesized to have evolved primarily to facilitate tissue remodelling during embryonic development, wherein it responds to specific developmental cues (105). In adult organisms, this mechanism is posited to have been adapted to enhance tissue regeneration and repair following injury; with a loss of senescent pathways exacerbating fibrotic responses (328, 329). Under normal conditions, this response upregulates cell-cycle arrest proteins and secretes a specific SASP to support regeneration and repair (328). Conversely, persistent cellular senescence is detrimental with AT2 cells becoming “locked in repair” in lung disease (330). Unable to re-enter the cell cycle, dysfunctional AT2 cells can promote senescence, apoptosis, aberrant repair mechanisms and inflammation via autocrine and paracrine signalling (103, 104, 331) (**Figure 1.4**). Cellular senescence of AT2 cells has been documented in both IPF and COPD (101, 102, 332).

Emerging research links iron accumulation to both cellular senescence and SASP (107, 119). *In vitro* studies indicate that iron levels accumulate over time due to a breakdown in cellular iron homeostasis, thus driving senescence (333, 334). Similarly, *in vivo* research shows that vascular or haemolytic injuries can lead to iron accumulation, which in turn promotes senescence and fibrosis (119). Supporting this, hereditary hemochromatosis, characterized by excessive iron deposition is also linked to senescence and fibrosis (335). This

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accumulation, coupled with senescent cells' resistance to ferroptosis (iron-dependent cell death pathway) suggests a strong link between the two pathologies (107).

However, cellular senescence is also linked with iron deficiency. *In vitro* studies involving iron chelation with DFX, have demonstrated that reducing iron levels induces a senescent-like growth arrest in human hepatocytes (278, 336). Additionally, Fanconi anaemia, a genetic disorder leading to reduced blood cell production and bone marrow failure, has been linked to cellular senescence (337). Furthermore, aplastic anaemia also underscores the connection between iron regulation and senescence; with bone marrow mesenchymal stem cells showing an enrichment of senescence-associated genes and pathways (338).

Therefore, cellular senescence can be triggered under conditions of both iron overload and deficiency, which is possibly serving as a stress response or checkpoint barrier for cells to determine their course of action. In lung disease this cycle is disrupted, where cells are thought to become persistently arrested driving hyperproliferation and repair in neighbouring cells through autocrine and paracrine SASP signalling (330). These cells are also suggested to evade immune clearance, resulting in the accumulation of senescent cells, thus contributing to lung pathology. Whether or not iron metabolism contributes to this 'locked' in repair phenotype remains to be explored.

In IPF, AT2 cellular senescence is characterized by the upregulation of cell-cycle arrest proteins such as *p53*, *p21*, and *p16*, alongside a SASP rich in pro-fibrotic growth factors, including *TGF-β* (101). This unresolved senescence in IPF leads to aberrant repair processes, fibroblast proliferation and excessive extracellular matrix deposition (331). In COPD, alveolar cell senescence in pulmonary emphysema is marked by the expression of *p21* and *p16*, where increased senescence correlates with greater airflow limitation (102). Conversely, in COPD, senescent AT2 cells are recognized for their inflammatory profile, which is thought to promote aberrant inflammatory responses in neighbouring immune cells, fostering a chronic inflammatory state that impedes tissue repair and enhances lung damage (99).

The existing literature on the direct impact of elevated pulmonary iron levels on AT2 cell dysfunction and senescence remains sparse. Recent studies using a murine BLM induced fibrosis model suggest that iron accumulates in AT2 cells, triggering ferroptosis [10]. Similarly, both acute and chronic exposure to CS in COPD models has been associated with AT2 cell damage and ferroptosis (209, 210). Of note, both BLM and CS are DNA-damaging agents,

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which could induce senescence independently of their relationship with iron. Furthermore, ferroptosis markers are investigated at the end-stages of these models, making it unclear whether iron accumulation in AT2 cells is a cause or a consequence of their dysfunction. Interestingly, intratracheal instillation of Fe^{2+} rapidly induces a fibrotic and senescent response within two days, suggesting iron may play a causative role (119). Moreover, co-treatment with iron chelators attenuates BLM-induced iron accumulation, further supporting a contributory role for iron in experimental fibrosis models [10]. Nonetheless, this increase in senescence and fibrosis could represent a 'normal' injury response to increased iron in the lungs, which could potentially resolve over time.

6.1.1 Chapter Aims and Objectives

Current research suggests a potential causative link between iron accumulation and AT2 cell dysfunction. However, this evidence is primarily derived from end-stage disease models or acute experimental conditions, which do not conclusively establish if iron dysregulation is a primary cause or a secondary effect of AT2 cell senescence and dysfunction. Notably, iron accumulation is consistently observed in senescent cells once cellular senescence has been established, indicating that iron accumulation may be a consequence of senescence (107, 119). Yet, the mechanisms leading to this accumulative state remain unexplored.

Previous research, detailed in **Chapters 4 and 5**, demonstrated that iron deficiency in AT2 cells induced through extracellular iron chelation or genetic deletion of mitochondrial iron transporters (*Mfrn1* and *Mfrn2*); leads to diminished AT2-cell specific functions. *In vivo*, AT2-specific iron deficiency disrupts lung iron homeostasis, driving inflammation and immune cell recruitment, which subsequently results in acute lung injury and fibrosis.

Building on these findings, this chapter aims to further delineate the temporal kinetics of iron dysregulation in lung disease models and assess whether cellular senescence in AT2 cells is initiated in the models described in **Chapters 4 and 5**. Utilizing CSE and BLM to model an aged alveolar epithelium *in vitro*, we will explore the role of iron in AT2 cell aging and dysfunction over time. Additionally, we aim to investigate how alterations in iron dynamics contribute to senescence and dysfunction of AT2 cells during the early stages of BLM induced fibrosis *in vivo*.

This chapter aims to;

1. Establish an *in vitro* model of the aged/injured lung epithelium observed in chronic lung diseases.
2. Elucidate the iron status in aged/injured MLE-12 cells during both acute and chronic stages of injury.
3. Investigate the impact of modulating intracellular iron and MITOIRON levels in AT2 cells on senescent markers.
4. Explore the changes in iron dynamics in AT2 cells in response to BLM during the early stages of BLM-induced fibrosis *in vivo*.

6.2 Results

Accumulating evidence suggests that AT2 cell dysfunction and senescence contribute to the pathogenesis of several chronic lung diseases (101, 102, 332). Concurrently, disrupted iron homeostasis has been implicated in IPF, with increased iron levels observed in the lungs of affected patients (114, 115). The relationship between iron dyshomeostasis and AT2 cell dysfunction remains poorly understood. Utilizing both *in vitro* and *in vivo* models, this chapter aims to elucidate the interplay between iron and AT2 cell dysfunction and senescence.

6.2.1 Establishing an *In Vitro* Model of the Injured Lung Epithelium Observed in Chronic Lung Diseases

To investigate iron dyshomeostasis in lung disease, we attempted to recapitulate an *in vitro* model established by Shaghaghi *et al.* (2021); who showed that MLE-12 cells exhibit characteristics of the aged epithelium when exposed to repeated low-doses of BLM (Figure 6.1A) (339). In this two-hit model, we observed significant loss of cellular viability, coupled with increased expression of cell cycle arrest proteins p21 and p53 (Figures 6.1B and 6.1C).

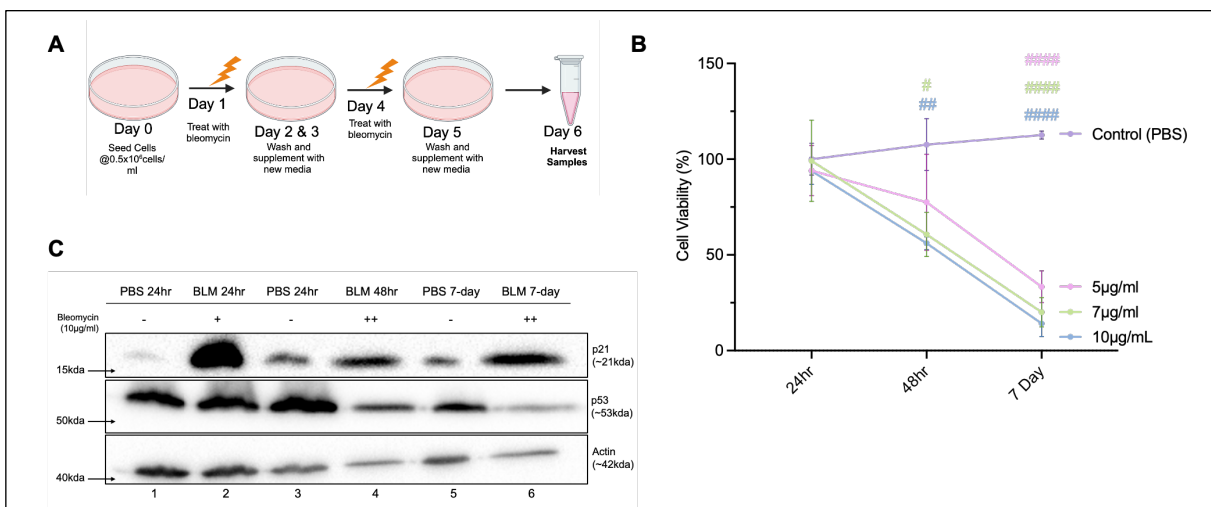
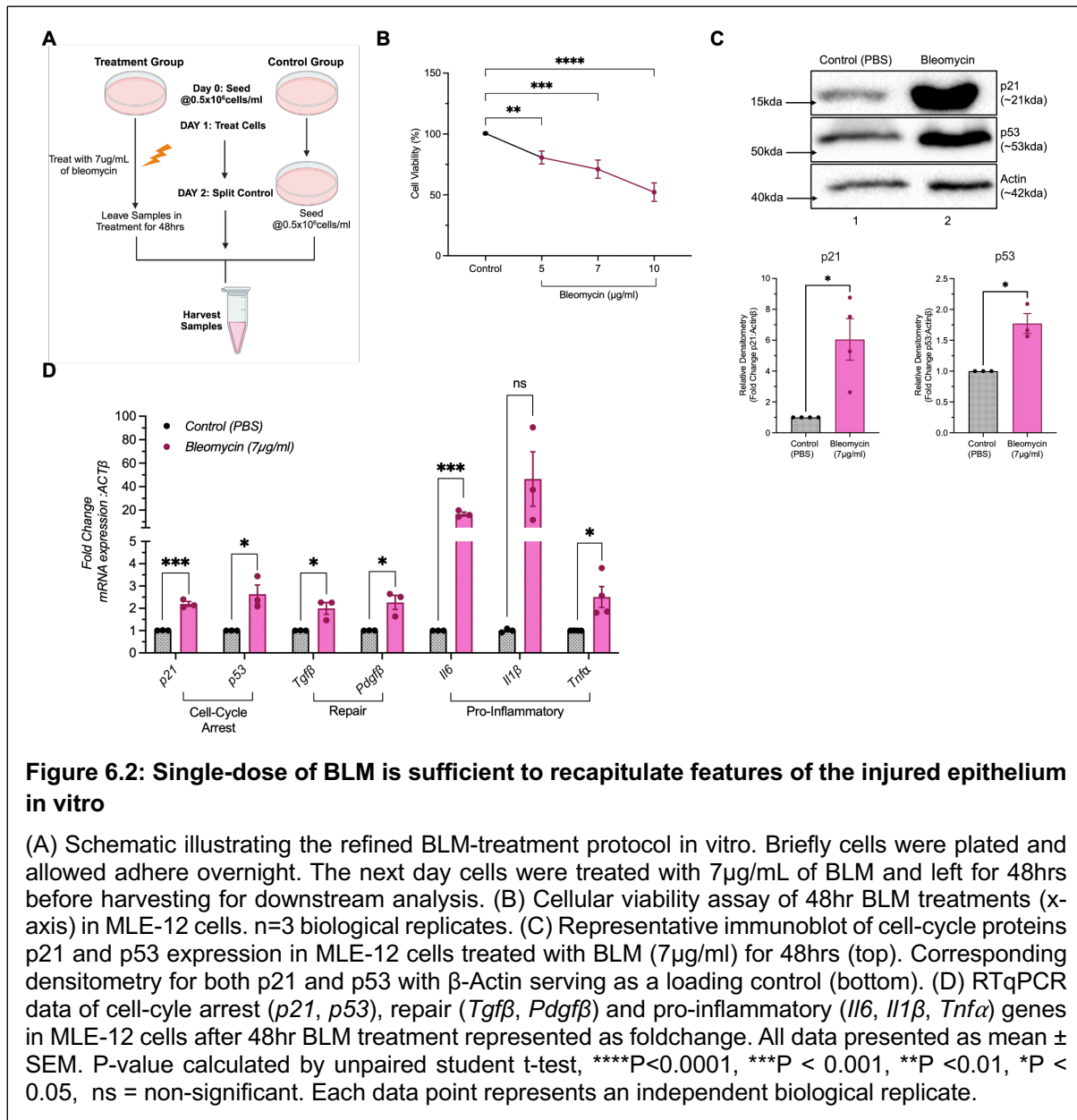


Figure 6.1: Multiple dosing of BLM upregulates cell-cycle arrest proteins in MLE-12 cells

(A) Schematic of the experimental design by Shaghaghi *et al.* (2021). Cells were plated on day 0, treated with 10 µg/mL BLM on day 1, then washed and supplemented with fresh media on day 2. On day 4, a second 10 µg/mL BLM treatment was applied, and cells were harvested on day 6. (B) Cell viability was assessed using alamar blue at 24hrs (after the first treatment), 48hrs (after two treatments), and 7 days (at the end of the experiment). (n=3 individual biological replicates). (C) Immunoblot of p21 and p53 expression in MLE-12 cells given multiple doses of BLM. (n=1). P-value calculated by ordinary one-way ANOVA dunnett multiple comparisons test, ##### P < 0.0001, ## P < 0.01, # P < 0.05, ns = non-significant. Significance between each group was compared to the control (PBS) at 24hrs, 48hrs and 7-days, colour of # corresponds to each treatment.

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Due to extensive cell death, the model was refined using a single-hit approach, treating MLE-12 cells with BLM for 48 hours (**Figure 6.2A**). This resulted in a dose-dependent decrease in viability (**Figure 6.2B**). At 7 $\mu\text{g/ml}$, we observed a significant upregulation of cell-cycle arrest proteins p21 and p53, at both protein and transcriptional levels (**Figures 6.2C and 6.2D**). Further characterization revealed that these cells exhibited a SASP, with elevated transcription of pro-fibrotic; *Tgfb* and *Pdgfb* and pro-inflammatory cytokines; *IL-6*, *IL-1 β* , and *TNF- α* (**Figure 6.2D**).



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BLM is commonly overlooked for its role as an iron chelator, forming complexes with iron to induce DNA strand breaks in both nuclear and mitochondrial compartments, acting as a ferric peroxide (340-344). Given this, we sought to develop an *in vitro* model of the injured epithelium, excluding the iron-mediated effects of BLM. To achieve this, we used cigarette smoke extract (CSE), an established risk factor for both IPF and COPD (84, 93), as an alternative method to model epithelial injury and damage.

Using a two-hit model, cells were exposed to 5% CSE for 24 hours, followed by a media change and an additional 5% CSE treatment for another 24 hours (**Figure 6.3A**). This regimen resulted in significant loss in cellular viability at 10% CSE after 48 hours, with minimal cell death after 24 hours (**Figure 6.3B**). Consequently, we chose to proceed with a two-hit dose of 5% CSE over 48 hours. Similar to the BLM model, CSE significantly induced the expression of the cell-cycle arrest protein p21 at both transcriptional and protein levels (**Figure 6.3C and 6.3D**). However, p53 protein expression remained unchanged, while its gene expression was significantly upregulated two-fold (**Figure 6.3C and 6.3D**). This suggests that the CSE two-hit model may be a less chronic model than the BLM 48-hour model. Additional gene expression profiling revealed a SASP phenotype characterized by increased expression of pro-fibrotic and pro-inflammatory markers (**Figure 6.3D**). In summary, our findings demonstrate that two doses of 5% CSE effectively replicate characteristics of injured epithelium *in vitro*.

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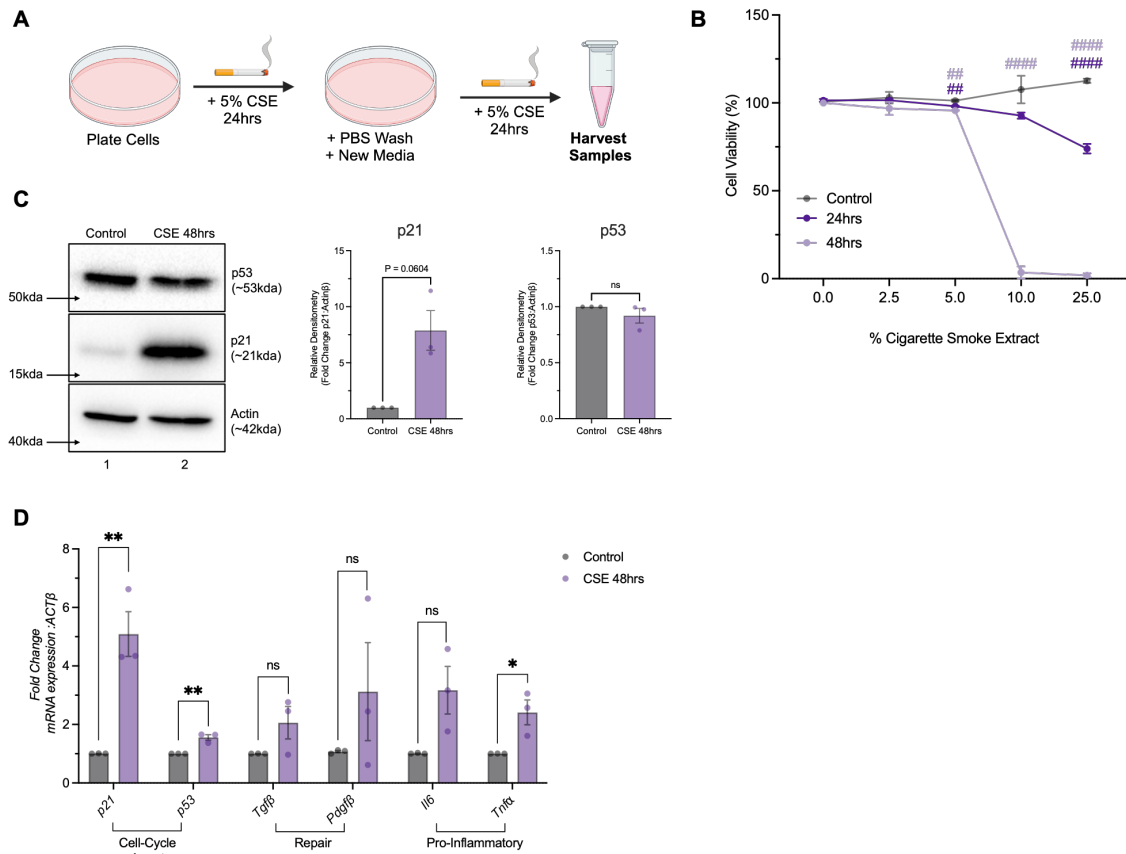


Figure 6.3: Prolonged exposure to CSE upregulates senescent and SASP markers in MLE-12 cells

(A) Experimental design for a two-hit CSE exposure model in MLE-12 cells. (B) Alamar Blue assay measuring cellular viability in MLE-12 cells treated with increasing % of CSE for 24hrs (grey) or two hits of CSE over a 48hr period (purple). n=2 independent biological replicates. (C) Representative immunoblot of cell-cycle proteins p21 and p53 expression in MLE-12 cells treated with two doses of 5% CSE over 48hrs (top) with corresponding densitometry for both p21 and p53 with β -Actin serving as a loading control (bottom). (D) RTqPCR data represented as foldchange of SASP markers in MLE-12 cells after prolonged 5% CSE exposure. SASP markers include; cell-cycle arrest (p21, p53), repair (*Tgf β* , *Pdgfr β*) and pro-inflammatory (*Il6*, *Il1 β* , *Tnf α*) genes. All data presented as mean $\pm$ SEM. P-value calculated by unpaired student t-test (*) or by ordinary one-way ANOVA dunnett multiple comparisons test (#), #####/****P<0.0001, ####/***P < 0.001, ###/**P < 0.01, #/*P < 0.05, ns = non-significant. Each data point represents an independent biological replicate. For panel (C) Significance between each group was compared to the control (untreated) at the given % of CSE, colour of # corresponds to each treatment.

6.2.2 BLM and CSE Treatment Drive MLE-12 Cell Dysfunction *In Vitro*.

After establishing our *in vitro* models, we investigated their impact on AT2 cell function. BLM treatment significantly reduced Pro-SFTPC protein levels, while CSE treatment had no effect (Figure 6.4A and 6.4B). For BLM treatment, there was a trend toward a decrease in the transcriptional expression of both *SftpC* and *SftpB* (Figure 6.4C). CSE significantly decreased *SftpC* transcript levels, while trending towards an increase in *SftpB* (Figure 6.4D). NBD-DPPC uptake remained unchanged in the 48-hour BLM model (Figure 6.4E), the most pronounced effect in the CSE model was a significant reduction in NBD-DPPC uptake (Figure 6.4F). These findings emphasize that distinct injury models differentially disrupt AT2 cell function, each affecting different aspects of surfactant homeostasis.

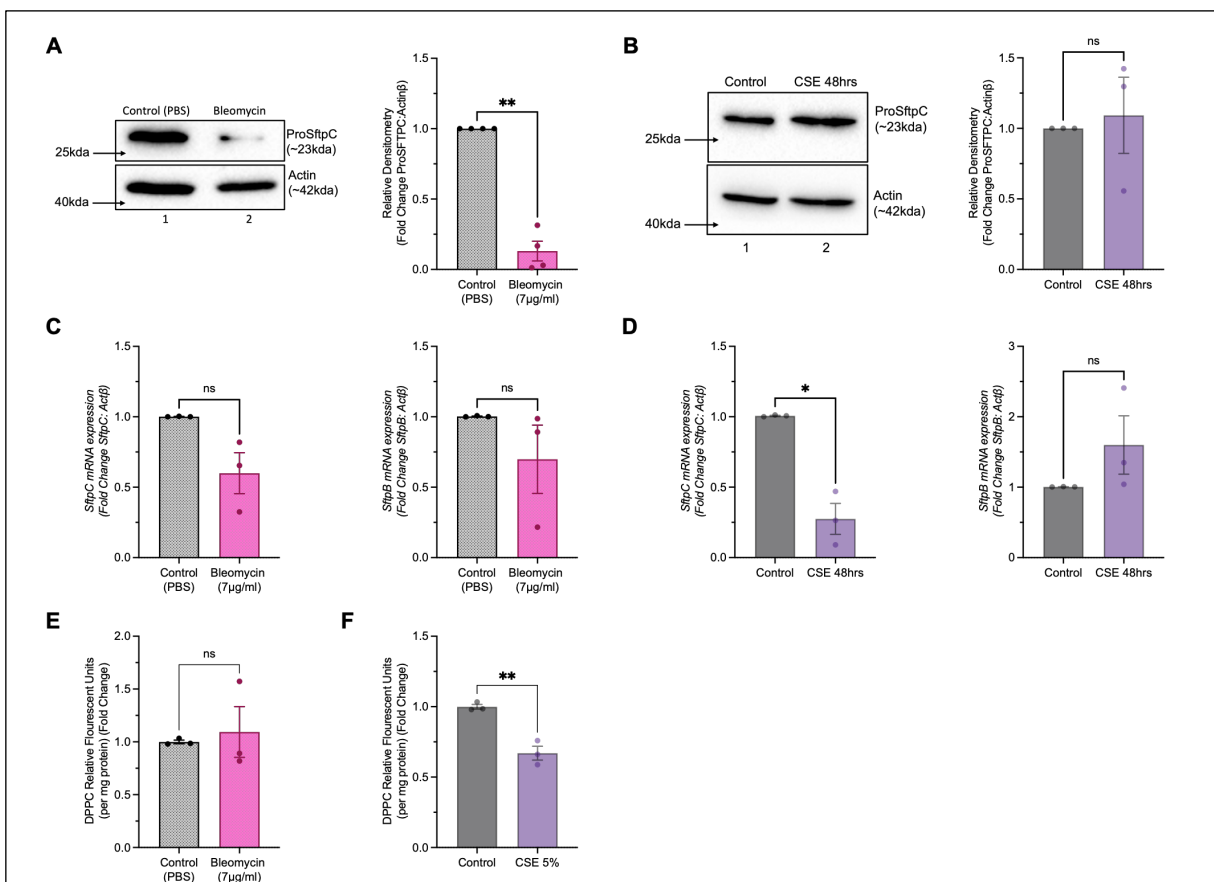


Figure 6.4: BLM and CSE differentially disrupt surfactant homeostasis in MLE-12 cells

Representative immunoblot of Pro-SFTPC (left) expression in MLE-12 cells treated with (A) 7µg/ml of BLM for 48hrs and (B) two-doses of 5% CSE for 48hrs compared to their relative control group. Corresponding densitometry with β-Actin serving as a loading control (right). RT-qPCR data of *SftpC* and *SftpB* in MLE-12 cells after (C) 48hr BLM model and (D) Two-hit CSE 48hr model. NBD-DPPC uptake normalised to protein (mg) represented as fold-change in (E) 48hr BLM-model treatment and (F) two-hit CSE 48hr-model. All data presented as mean ± SEM. P-value calculated by unpaired student t-test, **P < 0.01, *P < 0.05, ns = non-significant. Each data point represents an independent biological replicate.

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BLM treatment significantly impaired the wound healing capacity of MLE-12 cells, preventing wound closure after 48 hours (**Figures 6.5A - 6.5E**). BLM exposure, both before and after injury, completely halted cell growth and proliferation, with recovery similar to that of serum-free conditions (**Figures 6.5D and 6.5E**).

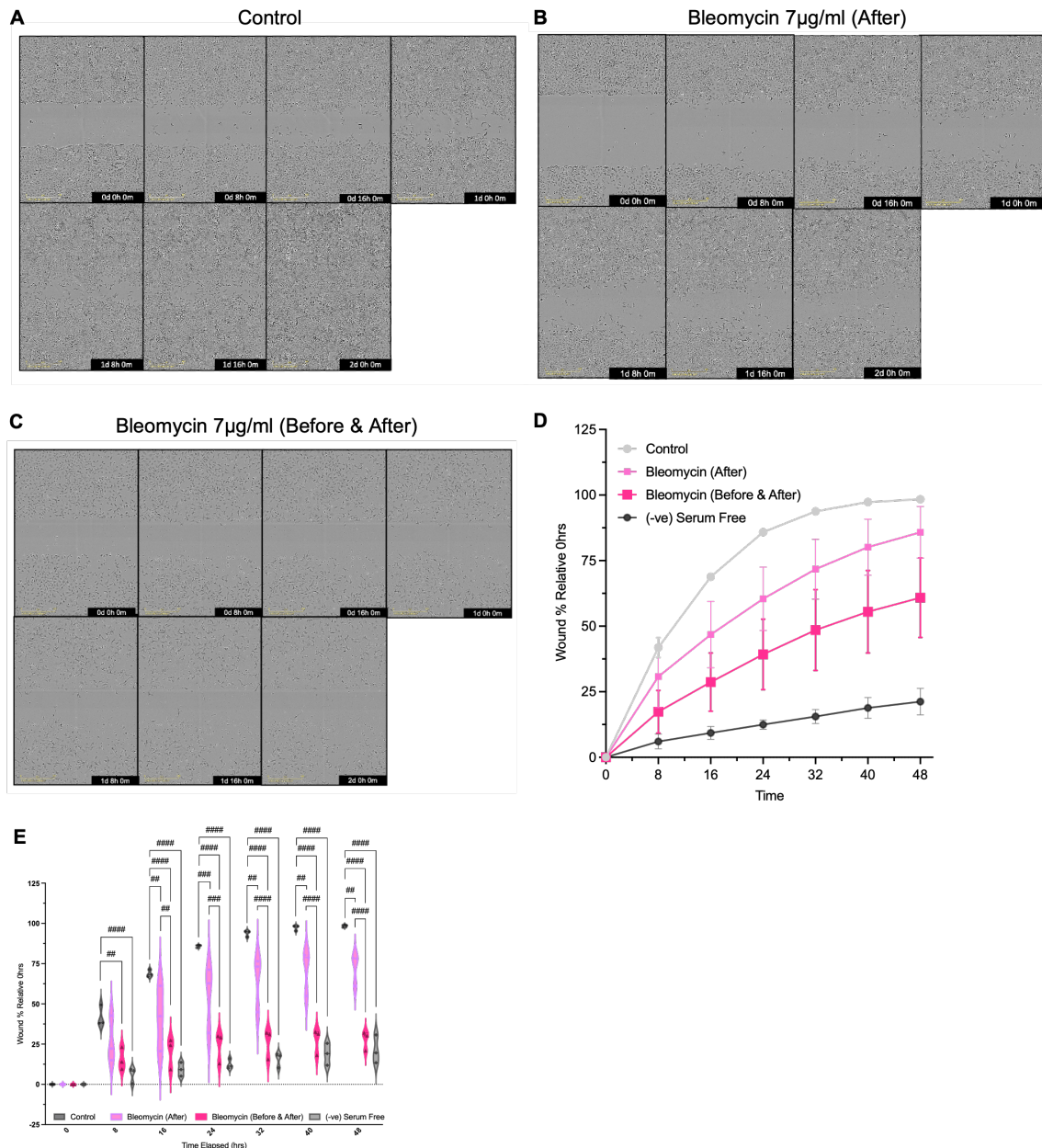
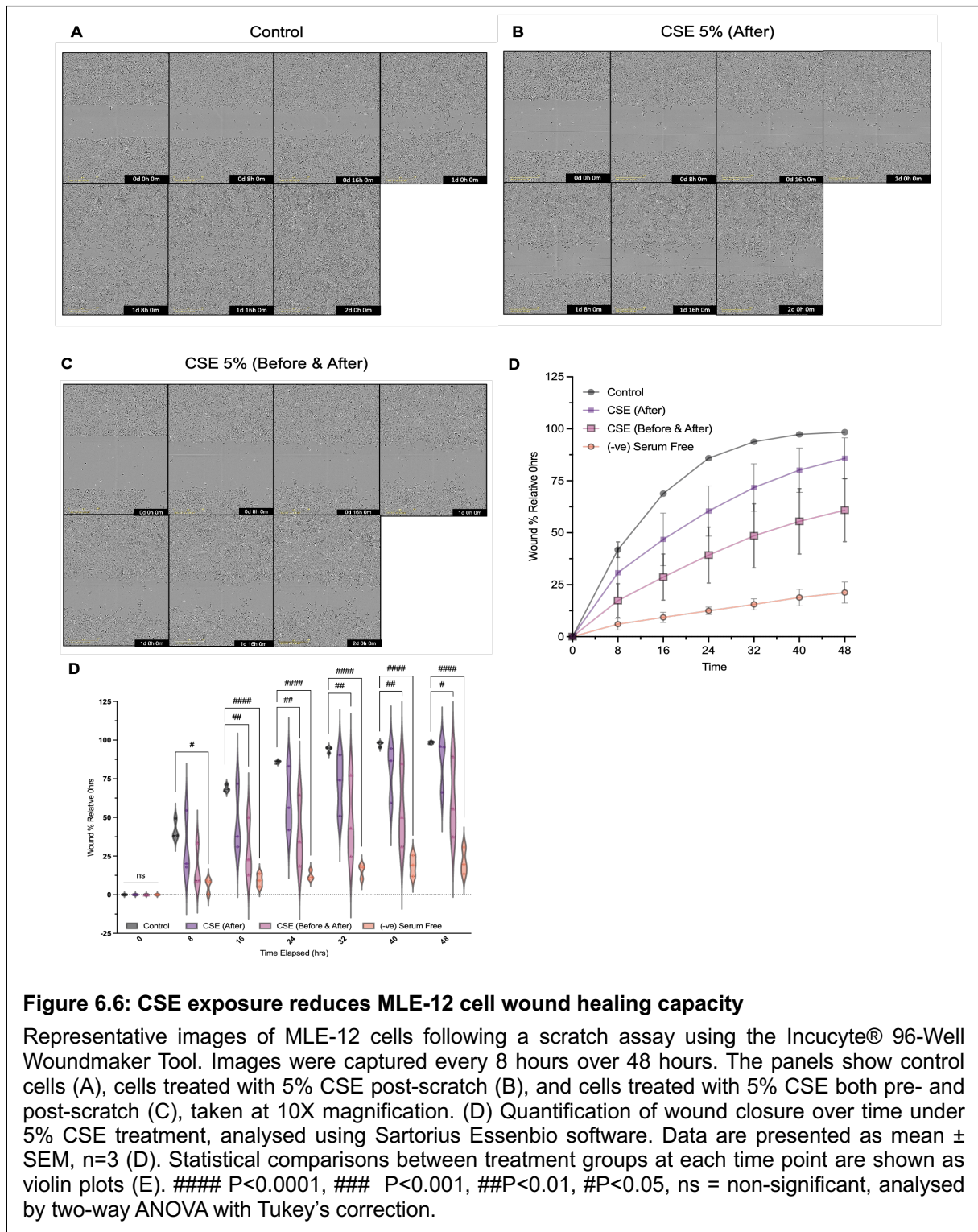


Figure 6.5: BLM significantly impairs MLE-12 cells repair capacity

(A) Representative images of MLE-12 cells following a scratch assay using the Incucyte® 96-Well Woundmaker Tool. Images were captured every 8 hours over 48 hours. The panels show control cells (A), cells treated with 5% CSE post-scratch (B), and cells treated with 5% CSE both pre- and post-scratch (C), taken at 10X magnification. (D) Quantification of cell migration and wound closure over time under 5% CSE treatment, analysed using Sartorius Essenbio software. Data are presented as mean $\pm$ SEM, $n=3$ (D). Statistical comparisons between treatment groups at each time point are shown as violin plots (##### $P<0.0001$, ### $P<0.001$, ## $P<0.01$, # $P<0.05$, analysed by two-way ANOVA with Tukey's correction).

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Likewise, CSE treatment severely hindered wound healing, with cells unable to recover within the timeframe (**Figures 6.6A - 6.6E**). While CSE treatment before and after injury also worsened the outcome, its effect was less severe compared to BLM.



6.2.3 Chronic Exposure to Both BLM and CSE Results in an Iron Overloaded Phenotype in MLE-12 Cells

Consistent with existing literature, our *in vitro* injured epithelium exhibits dysregulated iron metabolism (**Figure 6.7A – 6.7D**). Both BLM and CSE treatments result in a two-fold increase in intracellular iron levels measured by GF-AAS (**Figure 6.7A and 6.7C**). In the BLM model, there is a significant reduction in iron uptake and transport machinery (*Mfrn1*, *Mfrn2*, and *TfrC*), alongside increased expression of iron storage (*Fth1* and *Ftl*) and export (*Slc40a1*) pathways (**Figure 6.7B**), consistent with GFAAS data (**Figure 6.7A**). Similarly, the CSE model demonstrates reduced expression of iron uptake machinery and increased expression of iron storage and export pathways. However, mitochondrial iron transporters *Mfrn1* and *Mfrn2* are upregulated in this model (**Figure 6.7D**). Given that both models present with an iron-overloaded phenotype, this further solidifies the role of iron dysregulation in AT2 cell dysfunction.

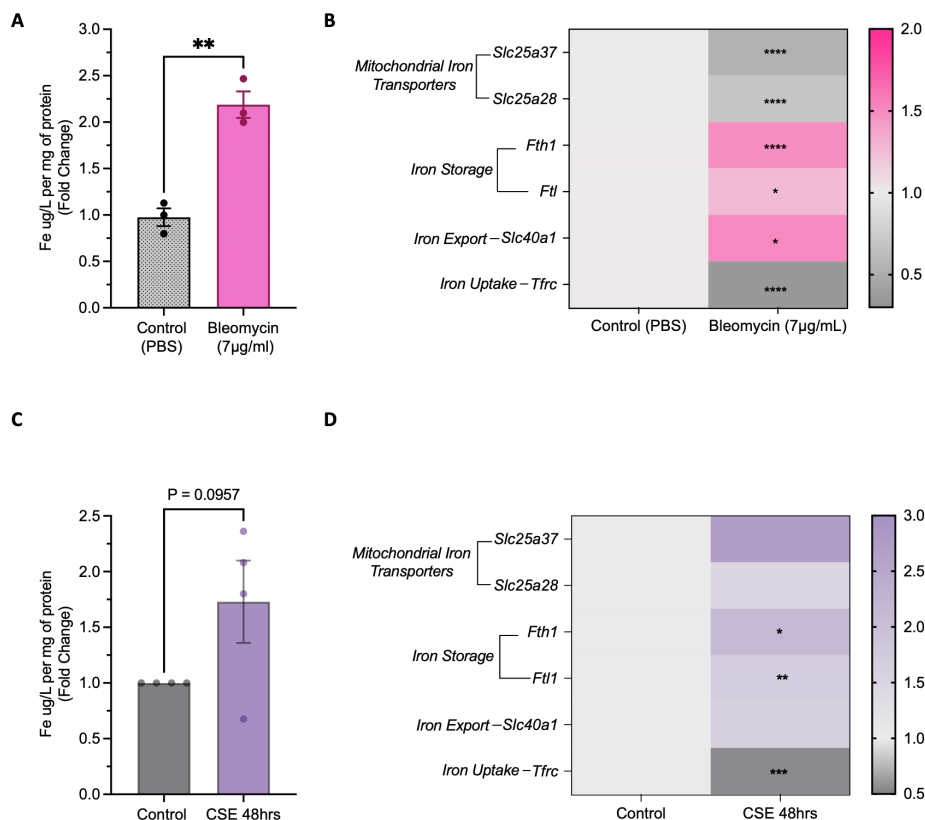
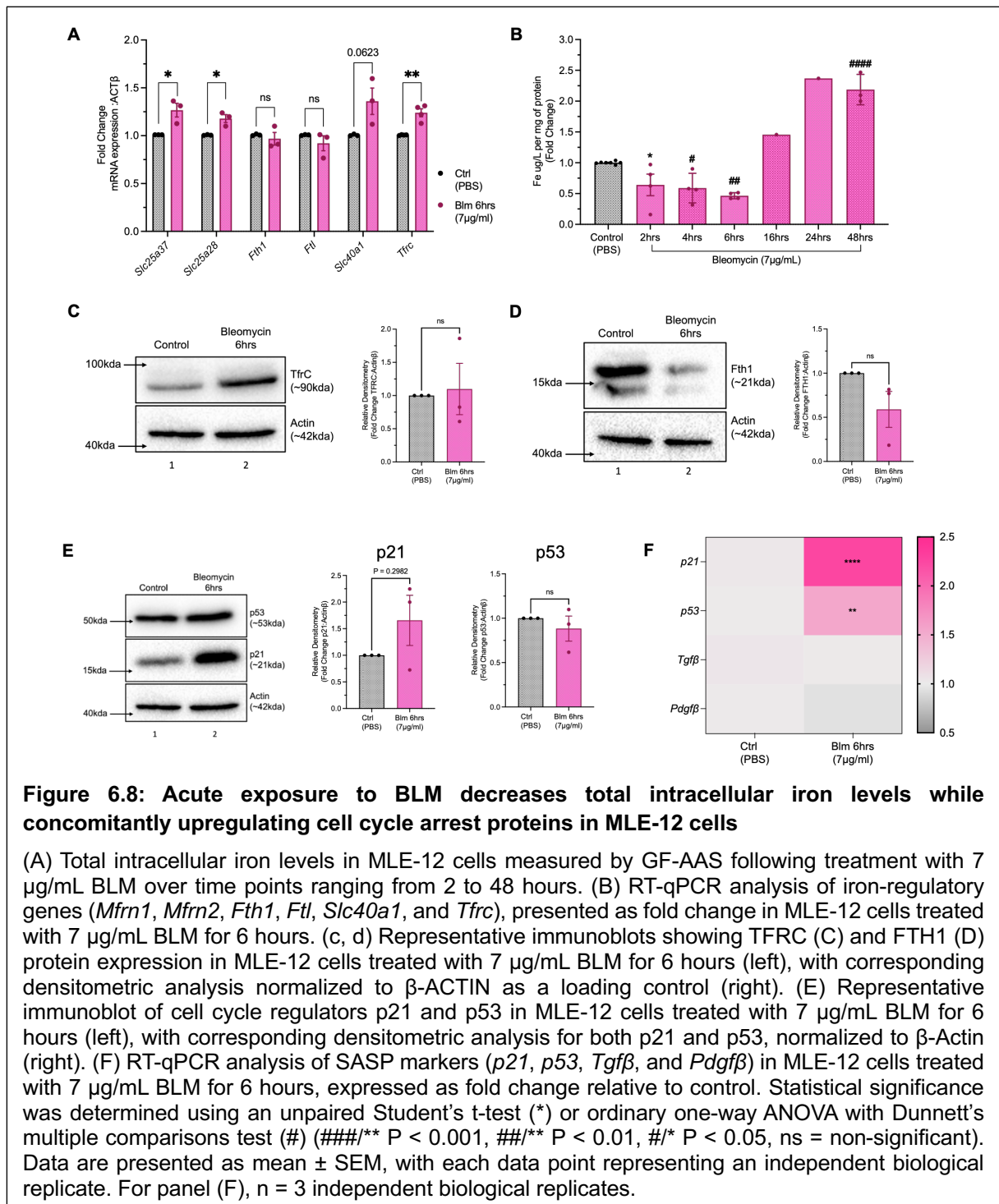


Figure 6.7: Prolonged exposure to both BLM and CSE increases total intracellular iron levels in MLE-12 cells

(A) Total intracellular iron levels in MLE-12 cells measured by GFAAS following 48-hour BLM treatment. (B) Relative mRNA expression levels of iron-regulatory genes (*Mfrn1*, *Mfrn2*, *Fth1*, *Ftl*, *Slc40a1*, *TfrC*), assessed by RT-qPCR in MLE-12 cells after 48 hours of BLM exposure. (C) Total intracellular iron levels measured by GFAAS in MLE-12 cells following a 48-hour two-hit CSE model. (D) RT-qPCR analysis of the same iron-regulatory genes in the CSE model. Statistical significance determined using an unpaired Student's t-test ($P^{***} < 0.01$, $P^{**} < 0.01$, $P^* < 0.05$)

6.2.4 MLE-12 Cells Present With an Iron Deficient Phenotype in an Acute Injury Model of the Injured Epithelium *In Vitro*

Following the observation of iron overload in MLE-12 cells after chronic exposure to both CSE and BLM, we aimed to model an earlier, acute stage of AT2 dysfunction to investigate iron dynamics in the onset of AT2 cell dysfunction and senescence. To achieve this, we established shorter, less chronic versions of both the CSE and BLM *in vitro* models discussed above.



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Analysis of intracellular iron levels using GF-AAS revealed a time-dependent decrease, with iron levels reduced by half within 6 hours of treatment (**Figure 6.8A**). Extending the time course showed a gradual re-accumulation of intracellular iron beginning at 16 hours, culminating in saturation at 48 hours (**Figure 6.8A**). Given the most profound significant iron depletion was observed at 6 hours we proceeded with this time-point for further analysis. Investigations into gene and protein expression of iron regulatory machinery further suggested an iron deficient phenotype in MLE-12 cells after 6hrs of BLM treatment (**Figure 6.8B – 6.8D**). Consistently, BLM treatment upregulated the expression of cell-cycle arrest proteins p21 and p53 at both the transcriptional and protein levels, as early as 6 hours post-treatment (**Figure 6.8E and 6.8F**).

In agreement with the acute BLM model, the shorter one-hit 24hr CSE model exhibited significantly reduced intracellular iron levels, as determined by GF-AAS (**Figure 6.9A**). This reduction was accompanied by a trend toward increased expression of mitochondrial and cellular iron uptake genes (**Figure 6.9B**), indicative of an iron-deficient phenotype. However, genes involved in iron storage and export were also upregulated (**Figure 6.9CB**), highlighting disrupted iron homeostasis in this model. TFRC and FTH1 protein levels remained unchanged (**Figure 6.9C**). Acute exposure to CSE resulted in a trended increase in expression of p21 and p53 at both transcriptional and protein levels (**Figures 6.9D and 6.9E**).

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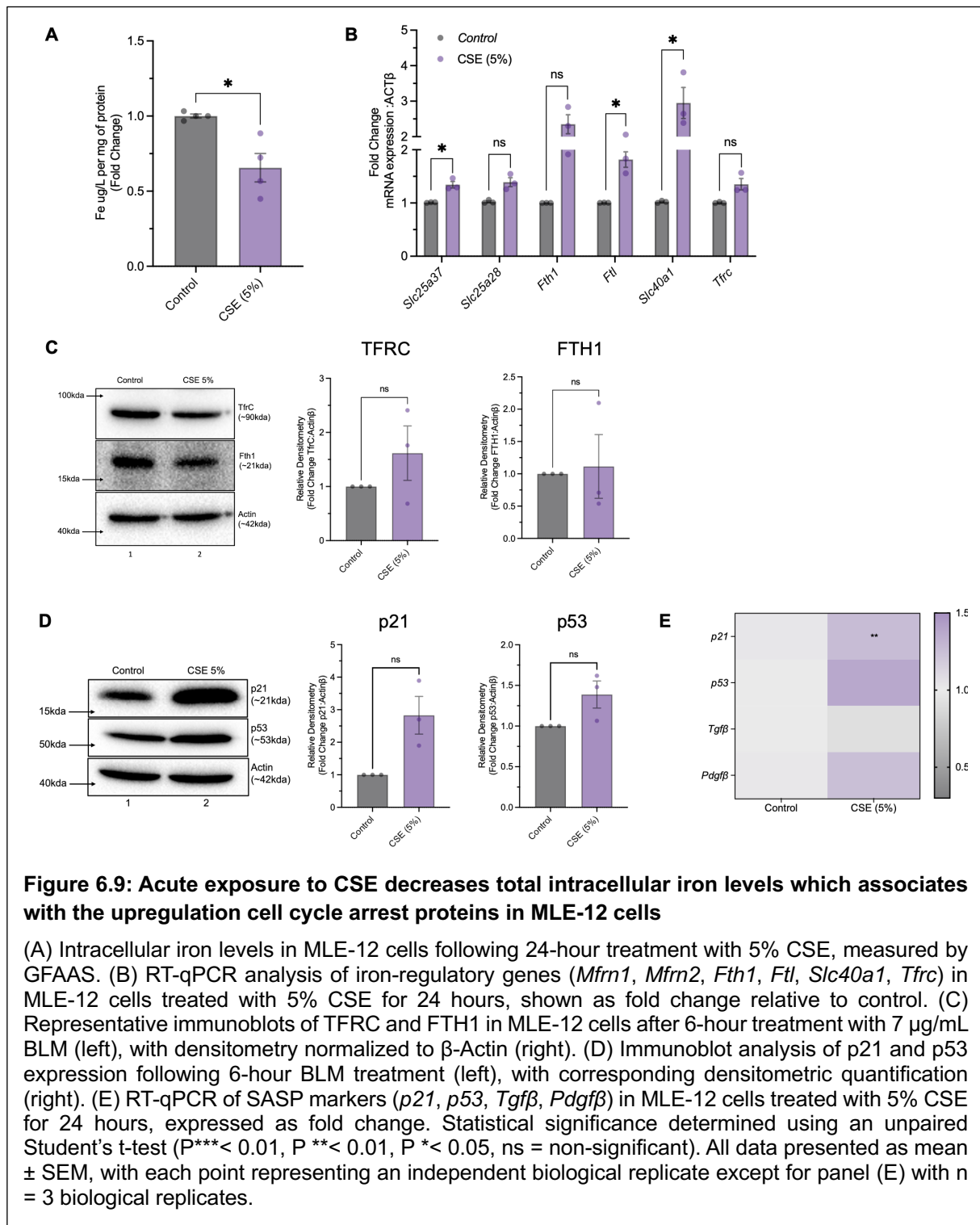


Figure 6.9: Acute exposure to CSE decreases total intracellular iron levels which associates with the upregulation cell cycle arrest proteins in MLE-12 cells

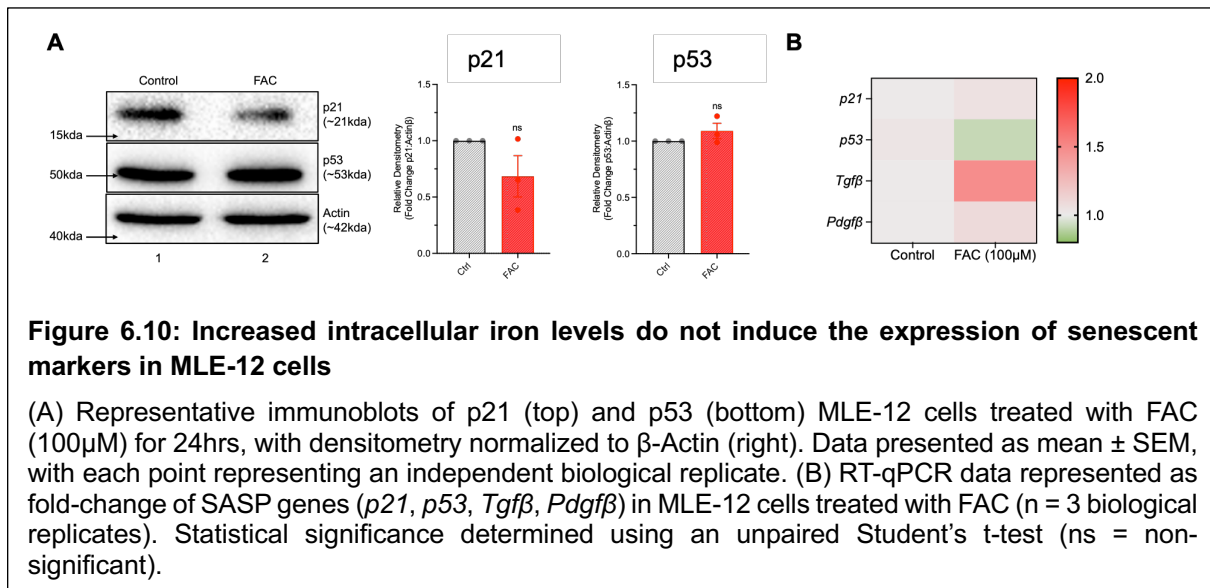
(A) Intracellular iron levels in MLE-12 cells following 24-hour treatment with 5% CSE, measured by GFAAS. (B) RT-qPCR analysis of iron-regulatory genes (*Mfrn1*, *Mfrn2*, *Fth1*, *Ftl*, *Slc40a1*, *Tfrc*) in MLE-12 cells treated with 5% CSE for 24 hours, shown as fold change relative to control. (C) Representative immunoblots of TFRC and FTH1 in MLE-12 cells after 6-hour treatment with 7 μ g/mL BLM (left), with densitometry normalized to β -Actin (right). (D) Immunoblot analysis of p21 and p53 expression following 6-hour BLM treatment (left), with corresponding densitometric quantification (right). (E) RT-qPCR of SASP markers (*p21*, *p53*, *Tgfb*, *Pdgfb*) in MLE-12 cells treated with 5% CSE for 24 hours, expressed as fold change. Statistical significance determined using an unpaired Student's t-test ($P^{***} < 0.01$, $P^{**} < 0.01$, $P^{*} < 0.05$, ns = non-significant). All data presented as mean $\pm$ SEM, with each point representing an independent biological replicate except for panel (E) with $n = 3$ biological replicates.

Taken together these findings suggest that during the early stages of injury, MLE-12 cells become iron-deficient state. Subsequently prolonged injury leads to iron accumulation, further exacerbating cellular dysfunction and driving the progression of injury-induced senescence and SASP.

6.2.5 Iron Deficiency in AT2 Cells Drives p21-Mediated Senescence Signatures *In Vitro* and *In Vivo*

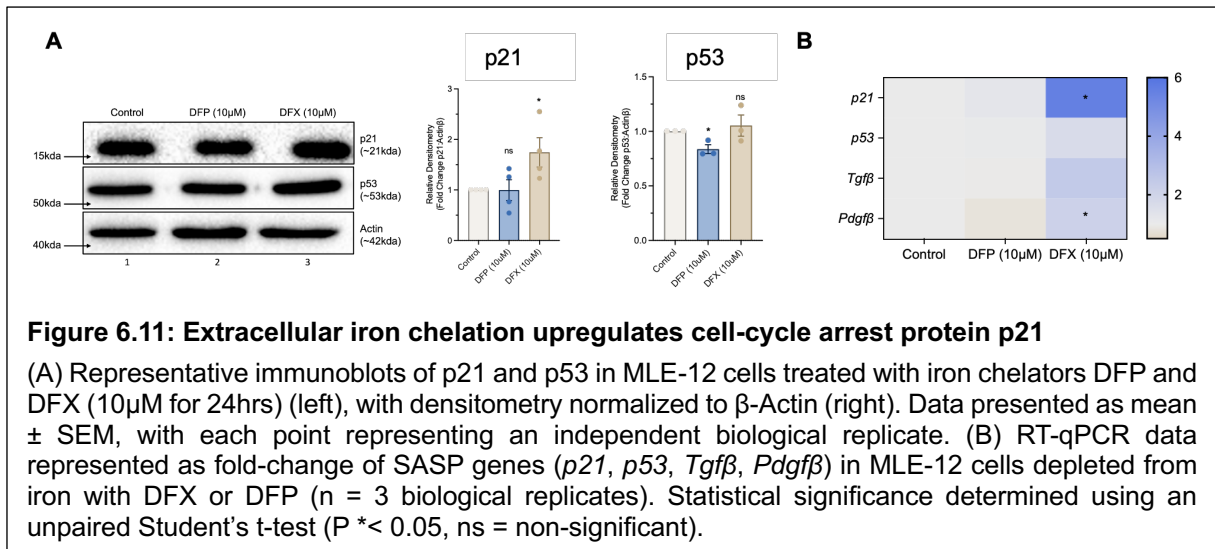
To further investigate how iron dynamics influence senescence, we examined the temporal expression of senescence markers in the models described in **Chapters 4 and 5**. Notably, iron-depleted cells, either through extracellular iron chelation (DFX) (*in vitro*) or impaired mitochondrial iron transport in AT2 cells (*in vitro* and *in vivo*) uniformly upregulated p21 expression.

Increased intracellular iron levels are known to impair cellular function and promote senescence (119, 334). Conversely, utilizing a more physiologically relevant iron dose (as described in **Chapter 4**) we demonstrate that a 24-hour treatment of MLE-12 cells with FAC downregulates the cell-cycle arrest protein p21 and does not alter SASP markers, aside for *Tgfβ* gene expression (**Figure 6.10A and 6.10B**).

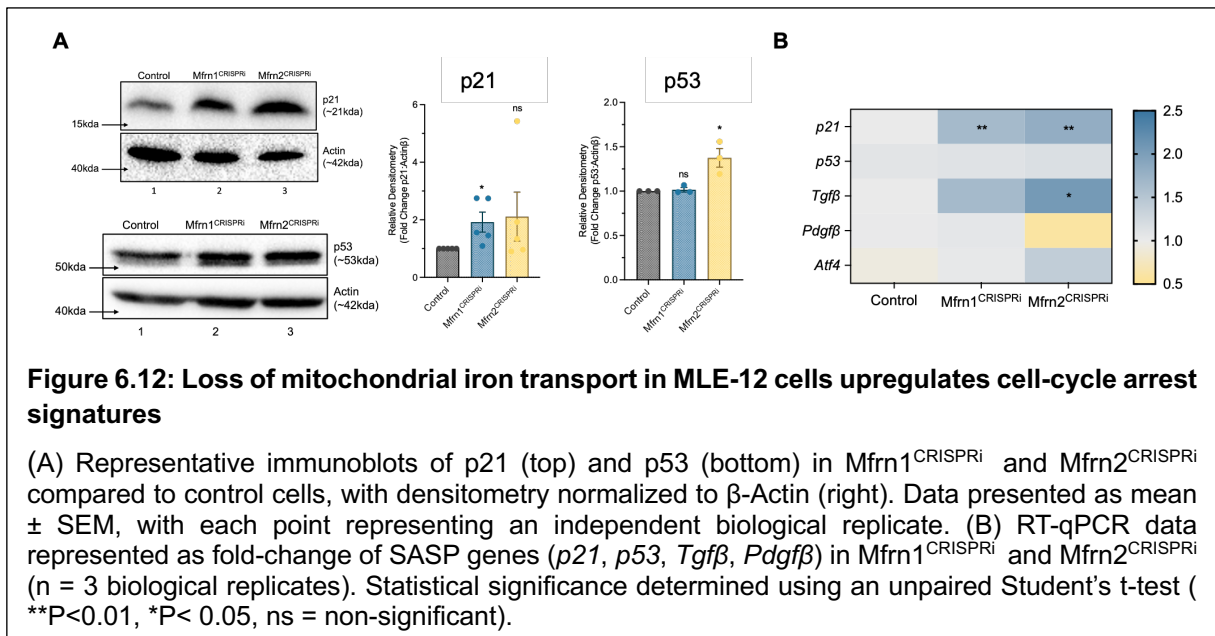


Intracellular iron depletion via DFP chelation did not affect senescence marker expression in MLE-12 cells (**Figure 6.11A and 6.11B**). However, extracellular iron chelation significantly upregulated p21 gene and protein expression (**Figure 6.11A and 6.11B**). Furthermore, there was a trend towards increased expression of additional senescence markers (*p53*, *Tgfβ*, and *Pdgfβ*) (**Figure 6.11B**).

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Notably, the loss of either *Mfrn1* or *Mfrn2* in MLE-12 cells, which results in significantly decreased intracellular iron and heme levels (**Chapter 4**), led to a more than two-fold upregulation of p21 expression at both the transcript and protein levels (**Figure 6.12A and 6.12B**). The loss of *Mfrn2* appeared to have a more profound effect, with significant upregulation of p53 protein levels, suggesting sustained induction of the p53-p21 axis (**Figure 6.12A**). Furthermore, *Mfrn2*^{CRISPRi} cells also showed significantly increased transcriptional levels of *Tgf-β* and trended increase in *Atf4* (**Figure 6.12B**).



Similarly, AT2 cells isolated from HetDKO and RDKO mouse models described in **Chapter 5**, also present with a similar cell-cycle arrest, stress and senescent signature. Bulk RNA-seq of hetDKO mice highlights a senescent phenotype, marked by genes involved in; growth arrest (*p21*, *Map2k1*), pro-inflammatory SASP signalling (*Icam1*, *Tgfβ*, *Tnfrsf11*) and tissue

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remodelling (*Mmp11*, *Mmp17*, *Timp2*, *Serpine2*) (**Figure 6.13A**). Gene expression analysis of AT2 cells from RDKO mice show a 6-fold increase in *p21* expression and a trend toward higher *p16* and *Atf4*, while *p53* expression is significantly downregulated (**Figure 6.13B**). Taken together, these results suggest that iron deficiency in AT2 cells promotes susceptibility to cellular stress and senescence, characterized by strong upregulation of *p21* and other stress markers, both *in vitro* and *in vivo*.

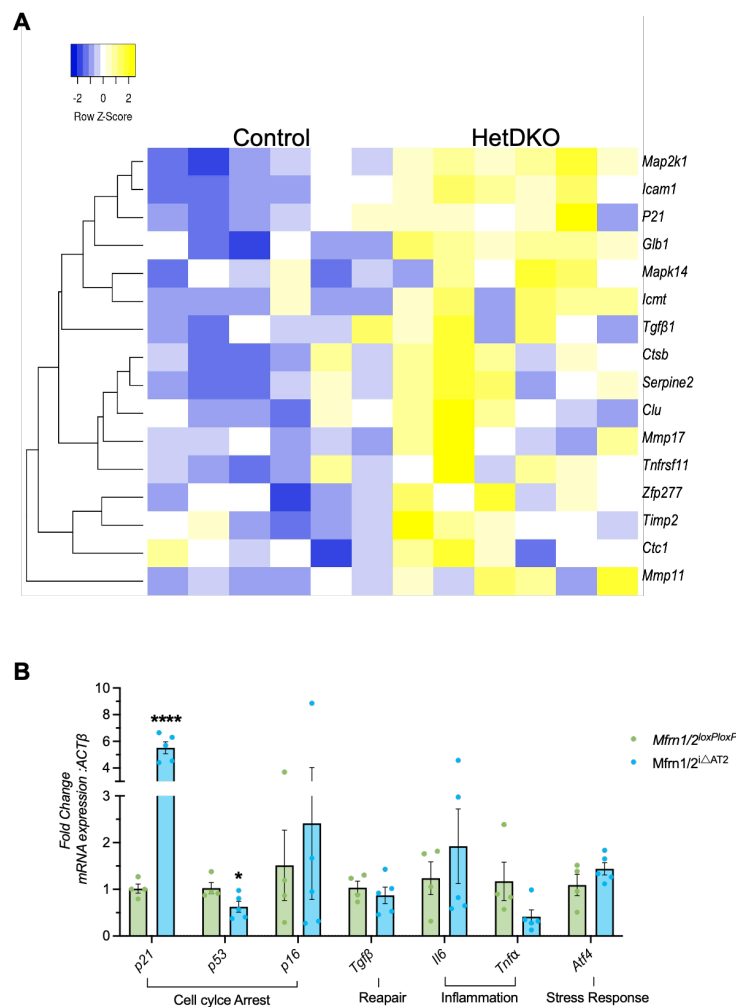
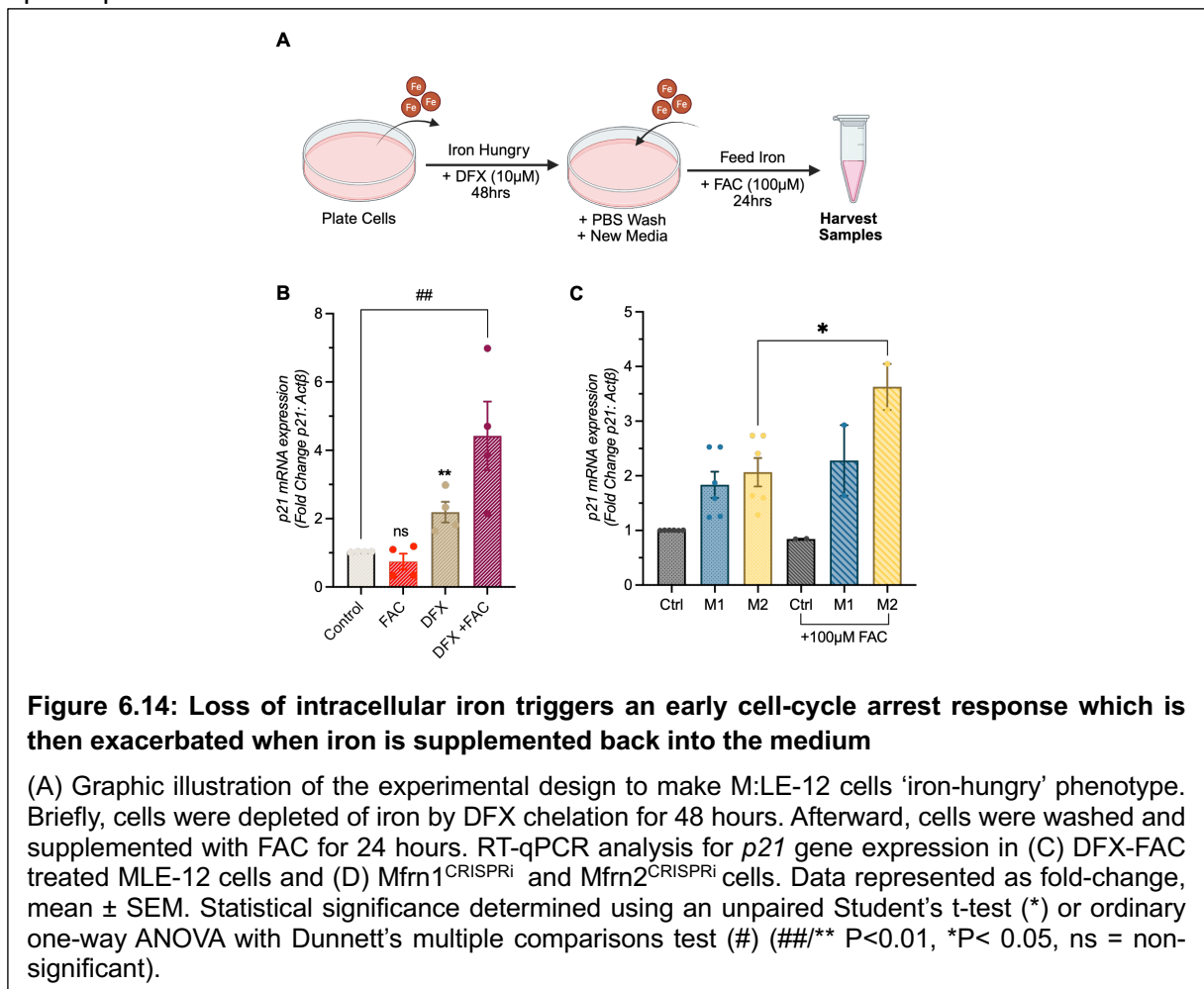


Figure 6.13: A loss of *Mfrn1* and *Mfrn2* specifically in AT2 cells associates with the upregulation of senescent and SASP markers in vivo

(A) Expression based heatmaps were generated with heatmapper.ca (1), from Bulk RNA-seq analysis of isolated AT2 cells from HetDKO mice. Plots show the upregulation (yellow) and downregulation (blue) of significantly altered senescent and SASP markers in AT2 cells isolated from HetDKO mice, under the annotation cellular senescence (GO:0090398). (B) RT-qPCR of senescent and SASP markers in AT2 cells isolated from RDKO mice. Data represented as fold-change, mean $\pm$ SEM. Each data point represents an individual mouse. P-value calculated by un-paired student t-test (**** $P < 0.0001$, * $P < 0.05$, ns = non-significant).

6.2.6 Iron Depletion Triggers Early Cell-Cycle Arrest, Initiating a Compensatory Iron-Acquisition Response Which Drives MLE-12 Cell Dysfunction

While iron accumulation has been implicated in senescence, our observations suggest that intracellular iron depletion triggers p21 upregulation. To further investigate the role of iron dynamics in the upregulation of the cell-cycle arrest protein p21, we explored the effects of extracellular iron chelation (DFX) followed by iron supplementation (FAC) (**Figure 6.14A**). Chelation alone significantly induced *p21* expression, while subsequent iron supplementation doubled this effect, resulting in a fourfold increase in *p21* gene expression (**Figure 6.14B**). We hypothesize that iron deprivation triggers cellular "iron hunger," leading to excessive iron uptake post-chelation.



Using CRISPRi MLE-12 cell lines with a loss in expression of *Mfrn1* or *Mfrn2* (shown in **Chapter 4** to exhibit reduced intracellular iron and heme levels); we observed elevated baseline *p21* expression (**Figure 6.12A and 6.12B**). Notably, *Mfrn2*^{CRISPRi} cells, but not *Mfrn1*^{CRISPRi} cells, further increased *p21* expression after 24 hours of FAC treatment (**Figure 14C**). These findings suggest that iron deficiency induces cell-cycle arrest in MLE-12 cells.

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Upon iron re-introduction, iron exacerbates *p21* expression and cellular dysfunction, highlighting the interplay between iron homeostasis and cell-cycle regulation.

6.2.7 Elucidating the Iron Status of AT2 Cells in Murine Lung Disease Models

After exploring how iron levels changed during the acute and chronic injury of MLE-12 cells and how depleting iron levels in these cells triggers cell-cycle arrest, leading to aberrant iron-sensing and initiating a compensatory, detrimental excessive uptake of iron. We wanted to extend these findings and investigate whether the same was true for *in vivo* models of injury. To explore this, I analysed existing data from isolated primary AT2 cells from a BLM-induced fibrosis murine model (developed by Ziling Huang at Weill Cornell Medicine). In this model, mice are intratracheally administered BLM (1 µg/g BW), leading to spontaneous lung fibrosis over a 21-day period (**Figure 6.15A**). Consistent with human fibrotic conditions, mice exposed

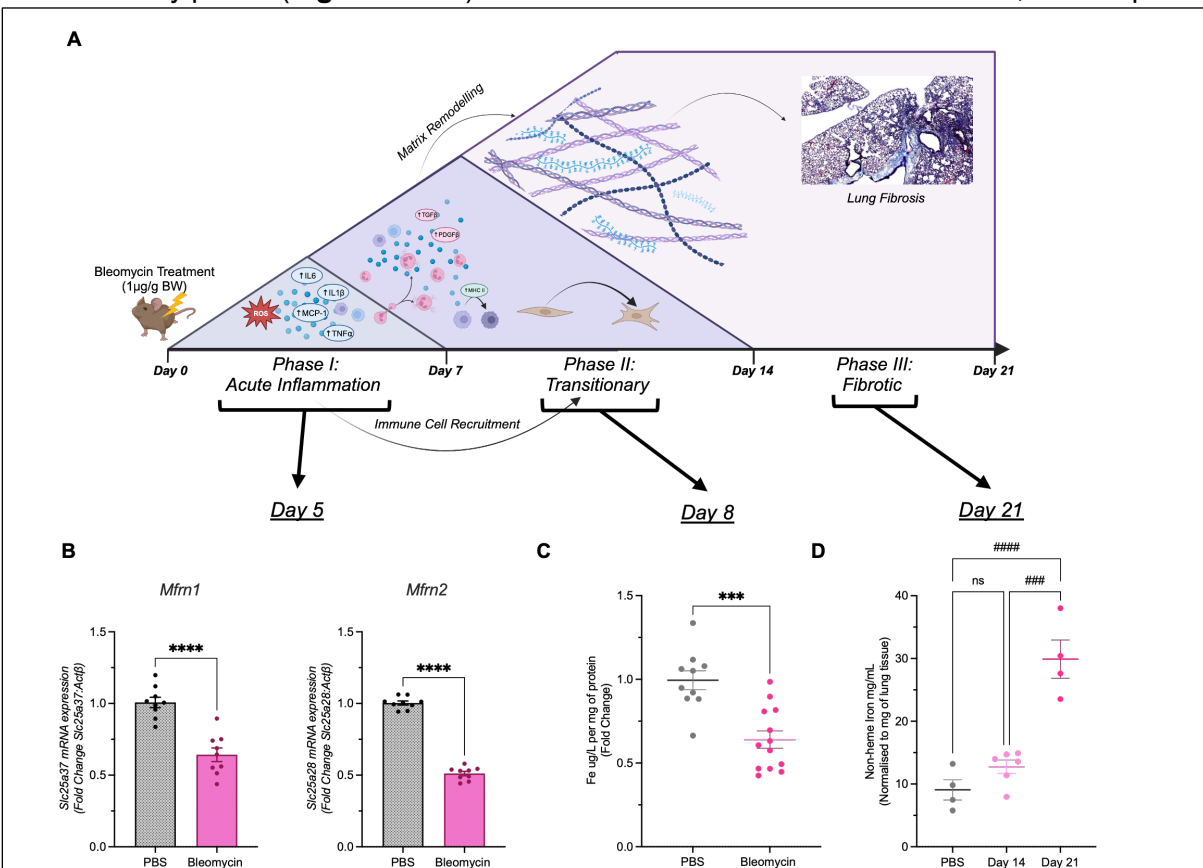
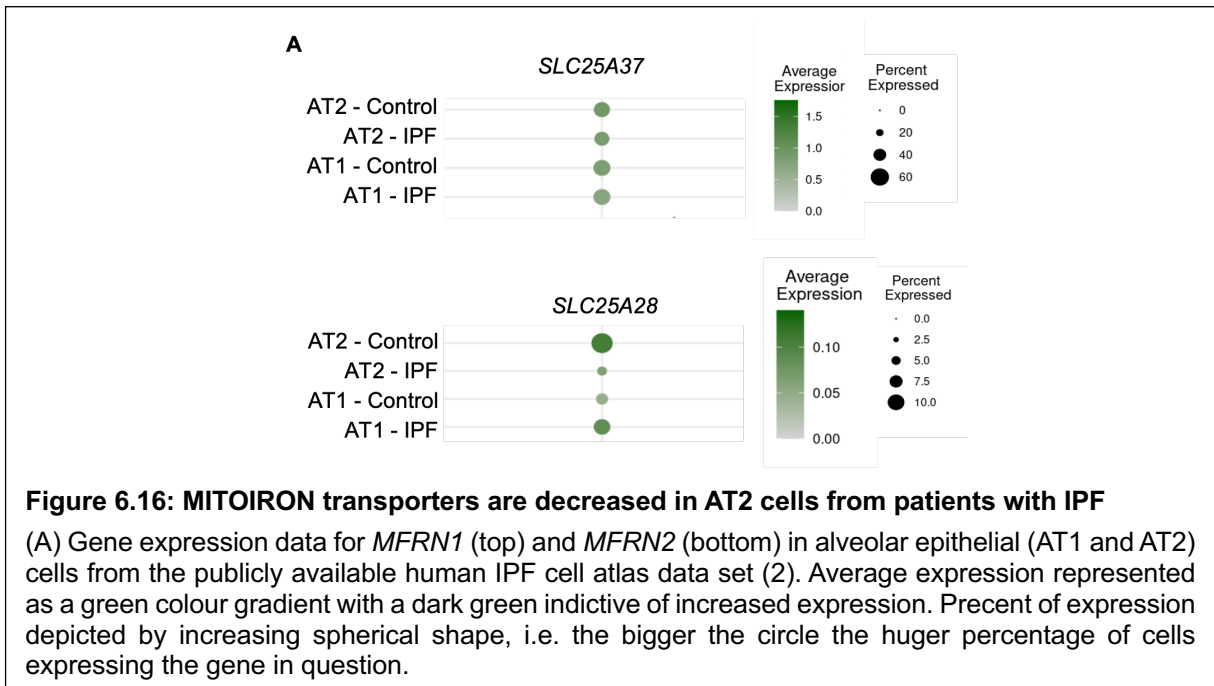


Figure 6.15: BLM provokes iron deficiency in AT2 cells, while driving lung-iron loading

(A) Illustration of the different stages of the 21-day BLM induced fibrosis murine model, see methods, section 2.3.2 Chapter 2. (B) Gene expression for *Slc25a37* (*Mfrn1*) and *Slc25a28* (*Mfrn2*) in AT2 cells isolated 5 days after instillation of BLM or vehicle control, PBS. (C) Total intracellular iron levels, measured by GFAAS, in AT2 cells isolated at day 8 from BLM treated and PBS treated mice. (D) Non-heme iron in lung tissue from BLM mice and their retrospective controls at day 0, day 14 and day 12. All data presented as mean $\pm$ SEM. P-value calculated by unpaired student t-test (*), or one-way ANOVA (#). #####/****P < 0.0001, ####/***P < 0.001, ns = non-significant. Each data point represents an individual mouse.

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to BLM show elevated lung iron levels compared to controls at day 21 (**Figure 6.15B**). However, interestingly, AT2 cells isolated during the acute inflammation/transitionary phase (days 5-8), show reduced intracellular iron levels and decreased expression of mitochondrial iron transporters *Mfrn1* and *Mfrn2* (**Figure 6.15C and 6.15D**). Furthermore, using the IPF Cell Atlas (2), we observed reduced expression of *MFRN1* and *MFRN2* in AT2 cells, with a more significant decrease in *MFRN2* (**Figure 6.16A and 6.16B**). Highlighting the potential importance of these transporters in disease pathogenesis of IPF.



Gene expression analysis of AT2 cells at Day 8 of the BLM-induced fibrosis model reveals significant alterations in the iron transcriptome (**Figure 6.17A**). Key upregulated iron-related genes include *Steap2*, *Steap3*, *Lcn2*, and *Tfr2*, indicating enhanced iron transport and uptake. Additionally, zinc transporters *Slc39a4* and *Slc39a14*, known to mediate iron uptake (138), are also increased. Notably, *Eik2ak1* (HRI), which inhibits protein synthesis during iron deficiency, heme deficiency or oxidative stress, is also upregulated; further solidifying the iron deficient phenotype for AT2 cells at this time point. This altered iron transcriptome associates with the upregulation of several senescent and SASP markers (**Figure 6.17B**). Isolated AT2 cells exhibit increased expression of genes associated with inflammation (*IL6*, *Ccl20*, *Cxc12*, *Cxcl3*, *Icam1*), ECM remodelling (*Col1a1*, *Col3a1*, *Fn1*, *Mmp2*, *Mmp14*, *Mmp15*, *Mmp28*, *Timp2*, *Ctsd*, *Ctsb*), and cell cycle regulation (*Pnpt1*, *Atr*, *Cdkn1A*, *Cdkn3*), which are all processes implicated in cellular senescence and SASP.

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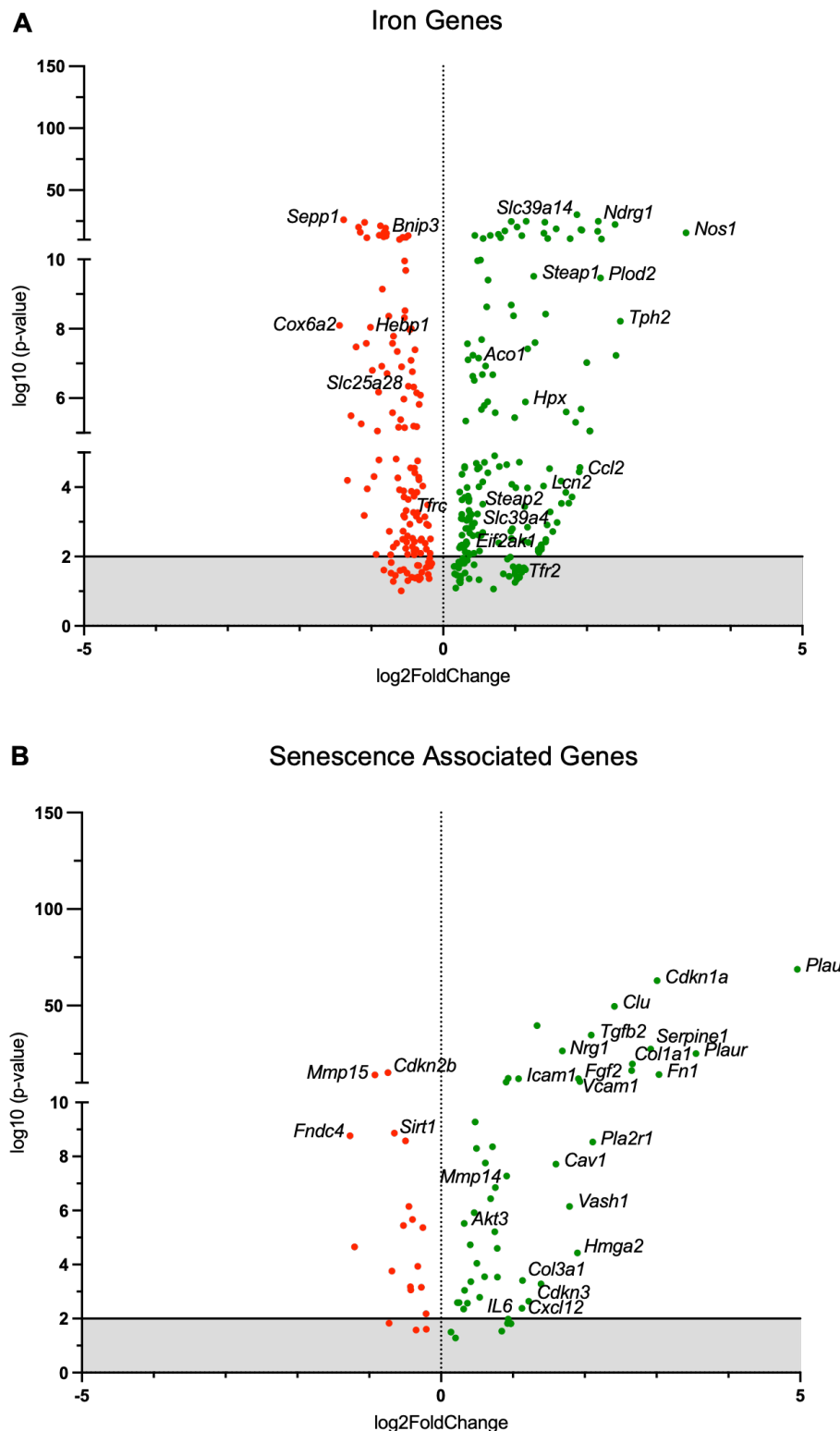


Figure 6.17: BLM alters the iron transcriptome in AT2 cells which associates with the upregulation of senescent markers

Volcano plot analysis illustrating significantly altered (A) iron genes and (B) senescence associated genes (GO:0090398) in AT2 cells isolated at day 8 from BLM-fibrosis compared to PBS-control. Threshold: adjusted p-value < 0.05, log2fold change > 2.

6.3 Discussion

It is evident from above, this chapter identified **a novel role** for iron depletion in promoting cellular senescence and subsequent iron accumulation in AT2 cells. Acute injury induces iron deprivation in MLE-12 cells, initiating pre-senescent stress response aimed at adapting to damage. However, sustained injury triggers a compensatory mechanism for iron uptake, exacerbating cellular stress and accelerating senescence. *In vivo* characterisation, demonstrated that AT2 cells isolated during the acute phase of BLM-induced fibrosis are both iron-deficient and senescent, highlighting a role for AT2-specific iron deficiency in BLM induced fibrosis.

A hallmark of cellular senescence is the upregulation of cell-cycle arrest proteins, which serve as check-points that halt proliferation, determining whether cells re-enter the cell-cycle or undergo apoptosis (103). In cancer biology, iron metabolism is a focal point of research due to its role in supporting unchecked proliferation, with iron chelators demonstrating anti-proliferative effects by halting cell-cycle progression (281).

Previous studies have shown that TFR1 expression peaks during the S-phase of the cell cycle, with siRNA-mediated silencing of *Tfr1* resulting in G1 arrest mediated by cell-cycle arrest proteins (345). Consistently, iron chelation has been observed to arrest cells specifically in the G1 phase (346). Investigations into the IRP/IRE network further solidifies the link between iron and the cell-cycle control, identifying a novel IRE-containing in cell-division cycle 14A mRNA (174). Our findings align with this, demonstrating that extracellular iron chelation using DFX, or intracellular iron depletion through loss of mitochondrial iron transporters *Mfrn1* and *Mfrn2*, leads to the upregulation of the cell-cycle arrest proteins p21 and p53. Notably, DFX treatment and *Mfrn1*-deficient cells specifically upregulate p21 independently of p53; suggesting a p53-independent pathway for p21 activation. Conversely, loss of *Mfrn2* triggers p21 and p53 activation suggesting p53-dependent mechanisms could be involved.

Similarly, iron chelation in hepatocytes induces markers of senescence and SASP, mediated through distinct cyclins; further reinforcing the role of iron deprivation in cellular proliferation arrest (278, 336). This senescent response has been attributed to impaired mitochondrial respiration resulting from deficiencies in Fe-S clusters, which constitute essential components of ETC complexes (278). Consequently, the observed senescent phenotype in MLE-12 cells lacking *Mfrn1* or *Mfrn2* could result from compromised Fe-S cluster biosynthesis, due to reduced intracellular and mitochondrial iron levels, therefore disrupting the formation of ETC complexes.

In agreement with this, AT2 cells from HetDKO and RDKO mice (**Chapter 5**) also exhibit increased expression of cell-cycle arrest proteins, with HetDKO significantly upregulating 16 senescence- and SASP-related genes. Cell-cycle arrest proteins are essential for modulating AT2 cell repair pathways. During injury, the cell-cycle arrest protein p21 is upregulated, suppressing WNT signalling (63, 64, 70). With sustained or repeated injury, p21 expression remains high, contributing to cellular senescence of AT2 cells observed in lung disease (64). P53 signalling promotes AT2-AT1 differentiation, with an AT2-specific loss of p53 in injury and lung cancer models leading to transitional cell proliferation and accumulation (63). Consistently, iron deficiency activates p21 expression in AT2 cells; via DFX chelation or a loss of mitochondrial iron transport. Aside from Mfrn2^{CRISPRi} cells, p53 is not activated in any of these models. Despite substantial AT2 cell damage and inflammation in RDKO mice, there is a notable decrease in p53 gene expression in these cells. As seen in **Chapter 5** AT2 cells from these mice exhibit reduced expression of AT2 markers and an increase in expression of transitional cell marker *Krt8*. With iron and iron metabolism being tightly linked to the cell-cycle (281), it is possible that sustained iron depletion disrupts 'normal' cell-cycle repair mechanisms in AT2 cells. Together this further supports the notion that disruptions in MITOIRON metabolism impact mitochondrial function, leading to the activation of pre-senescence, and disruptions in repair pathways. A key question that arises is what prevents these cells from re-entering the cell cycle and how iron contributes to this inhibition.

The current dogma considers that iron accumulates as a result of cellular senescence, which is exacerbated in fibrotic states (107, 119, 333, 334). *In vitro* experiments show that senescence inducers consistently increase intracellular iron levels, irrespective of the inducer used, confirming a common pathway of iron accumulation in cellular senescence. Masaldan *et al.* (2018) proposed that this is due to disrupted ferritinophagy, where senescent cells sequester iron in ferritin complexes, leading to increased iron uptake (334). In agreement, prolonged CSE and BLM exposure upregulates iron storage genes, however *Tfrc* expression is completely ablated after 48 hours. This is likely due to extensive damage or possible iron saturation (~2-fold vs. control). The expression of mitochondrial iron transporters (*Mfrn1/2*) increased during acute exposure to either BLM or CSE. However, with prolonged exposure, *Mfrn1/2* expression significantly decreased in the BLM model, while remaining elevated after 48 hours of CSE treatment. These opposing effects likely reflect differences in cellular iron loading, senescence, and mitochondrial stress. BLM induces substantial intracellular iron accumulation and likely mitochondrial iron saturation, triggering downregulation of *Mfrn1/2* as

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a protective mechanism. In contrast, CSE results in milder iron loading and a less pronounced senescence phenotype, possibly maintaining the need for mitochondrial iron import. Further studies measuring mitochondrial iron levels are needed to determine whether these transcriptional changes reflect shifts in mitochondrial iron homeostasis.

The study of senescence with *in vitro* models typically involves prolonged culturing, with senescence assessments conducted after five or more days (107, 119, 333, 334). However, little attention has been given to the role of iron and iron metabolism during the onset of prolonged cellular senescence in the 'injury' phase. A critical question remains: **"Is excessive iron a driver of senescence, or is its accumulation merely a downstream consequence of cellular senescence, wherein cells lose their ability to divide and redistribute iron?"**

Recent studies suggest that introducing iron, whether in the form of iron compounds (Fe^{2+} or Fe^{3+}), heme, or lysed red blood cells, is sufficient to trigger a senescent response across multiple different cell types (119). However, our findings do not indicate an increase in senescence or SASP markers following iron supplementation with FAC. Key distinctions include the use of physiologically relevant iron concentrations, which are threefold lower than those used by Maus *et al.* (2023) as well as differences in cell type and experimental duration.

Notably, to induce a senescent response, Maus *et al.* (2023) cultured cells under iron-deprived conditions (0.5% FBS) for 24 hours prior to iron supplementation (119). This represents a critical limitation, as iron depletion alone, confirmed by our findings and other studies (278, 336), triggers early senescence. The subsequent addition of iron under these conditions likely amplifies the senescent response. Our 'iron-hungry' model supports this observation, demonstrating that iron depletion-induced early senescence, followed by iron supplementation, leads to a significant upregulation of *p21*. We hypothesize that this occurs due to a compensatory response, in which iron-starved cells rapidly uptake iron, ultimately driving cellular senescence. Together this would suggest iron loading is the result of prolonged cellular senescence *in vitro*.

We next considered if the same was true *in vivo*. Extensive studies on fibrosis have demonstrated a strong correlation between iron accumulation and tissue injury in both experimental settings and human fibrotic diseases (119). Similarly, iron parameters are only considered after fibrosis is established, consequently, whether or not iron-related cellular mechanisms contribute to the onset of lung fibrosis remains unclear.

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In IPF, iron accumulation is primarily reported in AMs, with limited investigation into AT2 cells (115, 204, 207). Cheng *et al.* (2021) proposed that AT2 cells accumulate iron, leading to ferroptosis and promoting pulmonary fibrosis (208). However, their study measured ferroptosis markers in whole lung tissue at 21 days, rather than specifically in AT2 cells, limiting conclusions about AT2 cell iron status. Furthermore, this finding contradicts the established paradigm that senescent cells are resistant to apoptosis, with multiple studies confirming their resistance to ferroptosis (107, 334).

Here we demonstrate that during the early inflammatory stages of the BLM fibrosis model, AT2 cell iron levels are significantly reduced. AT2 cells also exhibit decreased expression of mitochondrial iron importers *Mfn1* and *Mfn2*, which are crucial for AT2 cell function and overall lung health (**Chapter 5**). Decreased iron levels associate with a strong upregulation of several senescence and SASP markers. Interestingly, a loss of both transporters results in a similar senescent profile *in vitro* and *in vivo*.

Although direct comparisons cannot be made, genetically induced anaemia in mice exacerbates BLM-induced fibrosis (347). Additionally, a study by Sato *et al.* (2020) demonstrated that an iron-deficient diet worsened CS-induced pulmonary inflammation in mice (290). Together this highlights the impact of an iron-deficient environment on the lungs response to injury.

Conversely, studies in hamsters subjected to iron deficiency through diet and bleeding have shown a reduction in BLM-induced pulmonary fibrosis (348). This aligns with the understanding that lower iron availability limits BLM's DNA-damaging effects, which are mediated through iron chelation, iron-catalysed oxygen radical formation and lipid peroxidation (340-344). Similarly, concomitant administration of DFX with BLM, followed by continuous DFX treatment attenuates fibrosis (208). Additionally, silencing airway hepcidin results in local cellular iron deficiency, thereby exacerbating the lung's response to injury and infection (194).

There is also debate regarding whether iron overload, induced genetically or through diet, exacerbates fibrosis. Some studies suggest a detrimental effect, while others report no significant impact. This inconsistency highlights the complexity of iron's role in pulmonary fibrosis and the need for further investigation into its precise mechanistic contributions, particularly at the cellular level.

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Given BLM's underappreciated role as an iron chelator (340-344), we hypothesize that its fibrotic effects stem from inducing iron deficiency in AT2 cells. In response, AT2 cells upregulate iron acquisition genes to compensate for iron loss, potentially leading to excessive iron uptake, senescence and the propagation of a fibrotic injury response in the lung. This phenomenon may align with the hypothesis that the aged epithelium behaves as though it is iron-deficient, a dysfunction that is exacerbated in disease states (267). Supporting this, recent findings indicate that AT2 cells from IPF patients exhibit an iron accumulation signature (119). Additionally, scRNA-seq studies of human IPF lung tissue reveal decreased expression of both mitoferrins in AT2 (2). However, the decreased expression of *Mfrn1/2* in AT2 cells from IPF patients may indicate either mitochondrial dysfunction with resultant iron deficiency or an excess mitochondrial iron.

Utilising the IPF cell atlas, scRNA-seq of lung explants from individuals with IPF suggests an iron-deficient phenotype in AT2 cells (**Figure 6.18**) (2). Increased expression of *IREB2* and

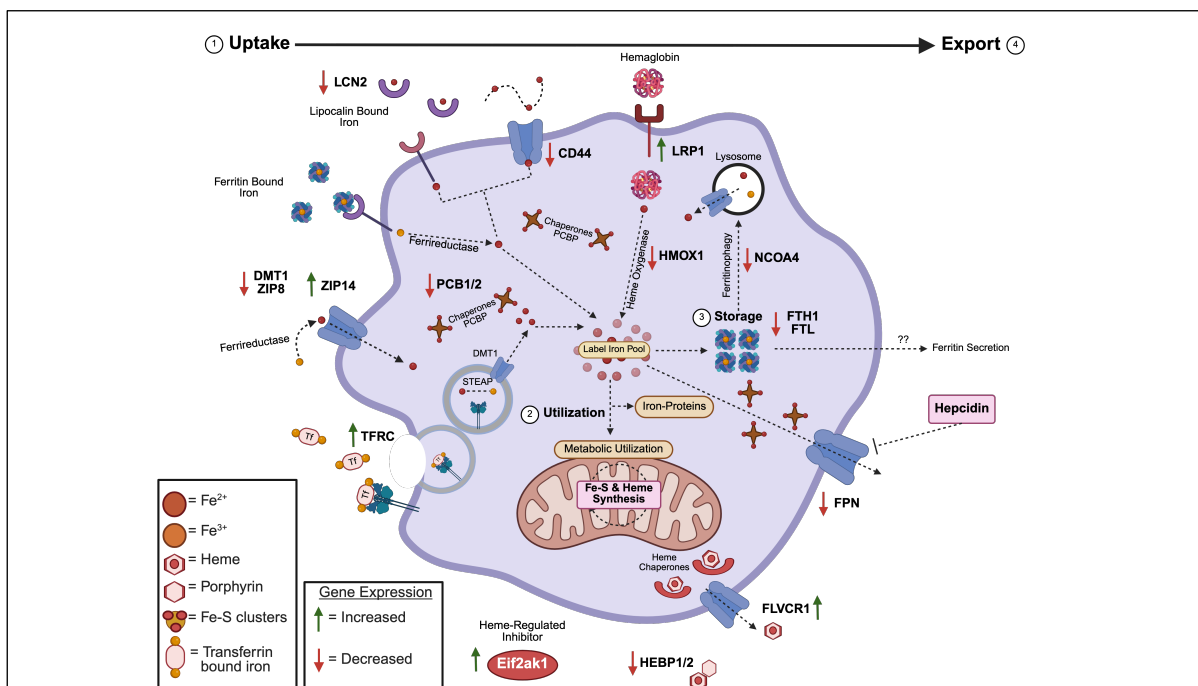


Figure 6.18: Cellular iron genes altered in AT2 cells from individuals with Idiopathic Pulmonary Fibrosis

Utilising the IPF cell atlas a webtool containing single cell RNAseq from eight control lung samples and eight pulmonary fibrosis explants, four of which were explants from individuals with IPF (2). Genes involved in cellular iron metabolism were explored in AT2 cells in attempt to understand the iron status of the cell. Green arrow = increased gene expression, red arrow = decreased gene expression.

EIF2AK1/HRI suggests the cells are responding to decreased iron levels. *IREB2* binds to IREs in storage genes (*FTH1* and *FTL*) in this case downregulating them while promoting iron uptake genes (*TFRC*), as observed. Elevated *EIF2AK1/HRI* further indicates intracellular heme and iron deficiency. However, RNA-seq data may not fully reflect protein levels, so direct

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measurement of intracellular iron in isolated AT2 cells is needed to clarify these regulatory changes. Together the above demonstrates a significant trend throughout which suggests a critical role for AT2-specific iron dyshomeostasis in the progression of lung injury and fibrosis.

6.4 Limitations

In this chapter, we identify a novel role for iron depletion in promoting AT2 cell dysfunction in both *in vitro* and *in vivo* models of the injured epithelium. However, several limitations must be considered when interpreting these findings.

Firstly, incorporating classical senescence inducers, such as doxorubicin or hydrogen peroxide, would have provided valuable controls for assessing iron dynamics across different senescence pathways. Similarly, utilizing a fibrotic cocktail for *in vitro* models could determine whether AT2 cell iron dysregulation is consistent across multiple aging and fibrotic stimuli, and not just CSE and BLM. Expanding senescence characterization beyond p21 and p53 to include markers such as β -galactosidase, p16, mitochondrial dysfunction, telomere attrition or DNA damage indicators would strengthen the validation of the senescent phenotype.

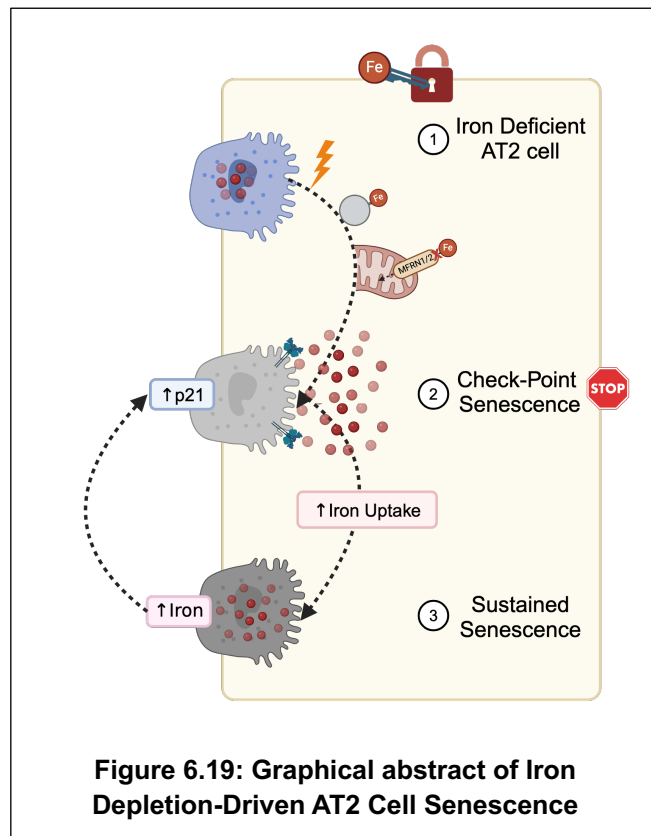
Additionally, this study lacks a longitudinal perspective on iron dynamics. Investigating how iron levels fluctuate during extended culture or following injury resolution (e.g., after removing BLM or CSE exposure) would clarify whether AT2 cells can recover, re-enter the cell cycle, or remain in a persistent senescent state. Previous studies using iron chelators in BLM-induced fibrosis or experimental (CS) COPD models suggest iron deposition in the lung can be alleviated, improving lung pathology (118, 208), raising the question of how AT2 cells respond to such interventions.

As previously noted, BLM is widely used to induce pulmonary fibrosis but does not fully recapitulate human disease. It chemically induces DNA damage, leading to senescence, whereas human fibrotic diseases involve multifactorial triggers such as oxidative stress, mitochondrial dysfunction, inflammation, and telomere attrition. Furthermore, its role as an iron chelator presents a significant limitation in studying iron dynamics in fibrosis development, as it directly alters iron metabolism. Evaluating iron dynamics in AT2 cells in alternative models, such as experimental (CS) COPD models or TGF- β -driven fibrosis, could provide broader insights into iron dynamics in disease progression. These findings underscore the need for caution when extrapolating from BLM-induced fibrosis to human pathology.

6.5 Conclusions

In summary, this chapter identifies iron depletion as a novel driver of AT2 cell dysfunction and senescence. We demonstrate that intracellular iron loss, whether through iron chelation or impaired mitochondrial iron transport, is associated with the upregulation of cell-cycle arrest proteins, as a protective check-point senescence response. We propose that iron deficiency triggers a compensatory 'iron-hungry' response, potentially leading to excessive iron acquisition, exacerbating cellular stress and driving senescence.

Interestingly, acute injury to AT2 cells induces a similar iron-deplete phenotype resulting in a protective check-point senescent response in both *in vitro* and *in vivo* models of epithelial cell injury. However, in the literature the complex relationship between iron levels and fibrosis progression remains incompletely understood, with both iron deficiency and iron overload showing context-dependent effects in different experimental models. Together, these findings suggest that AT2 cells may adapt their ironome in response to injury, but persistent injury disrupts this response, leading to iron accumulation and prolonged cellular senescence.



In summary, our study identifies iron deficiency as one possible initial trigger for cellular senescence and propose negative feedback loop involving excessive iron uptake as a key driver of sustained cellular dysfunction and senescence in AT2 cells (**Figure 6.19**).

7. Discussion

AT2 cells play a critical role in maintaining alveolar integrity, demanding substantial metabolic energy to fulfil their specialized functions. Consequently, AT2 cell loss or dysfunction is implicated in various chronic lung diseases. Mitochondria within AT2 cells are central to meeting these significant energy demands and require diverse nutrients for optimal function. Among essential mitochondrial nutrients, iron is particularly critical, reflecting a mutual dependence whereby mitochondria and iron homeostasis influence one another. Notably, disturbances in both mitochondrial function and iron regulation are associated with chronic lung diseases. Yet the role of iron and MITOIRON in regulating AT2 cell function remains unclear, presenting a significant gap in knowledge.

Given the challenges associated with studying AT2 cell function, the initial objective of this thesis was to devise a comprehensive, multimodal, and cost-effective methods for investigating these cells *in vitro* and *in vivo*. Utilizing the newly developed AT2 cell tool-kit, the **primary aim** of this thesis was to explore the impact of iron, including MITOIRON, on AT2 cell function in both health and disease.

7.1 Thesis Summary and General Discussion

The **primary finding** of this thesis establishes a pivotal role for iron across all aspects of AT2 cell specialised functions. Intracellular iron levels within AT2 cells must be carefully regulated, as deviations - either excess or deficiency - adversely affect their specialized functions. Most notably, the most profound impacts were observed when MITOIRON metabolism was genetically hindered, both *in vitro* and *in vivo*. For **the first time**, this study underscores the indispensable role of MITOIRON metabolism in maintaining surfactant homeostasis, AT2-derived signalling, and alveolar repair.

Chapter 4 demonstrated that both iron loading and iron deficiency adversely affect AT2 cell functionality in distinct ways. Specifically, excessive intracellular iron impaired wound healing capacity in MLE-12 cells, coupled with significantly reduced surfactant protein synthesis and lipid uptake. Interestingly, iron dynamics associated with surfactant secretion revealed increased intracellular iron levels during active secretion, suggesting increased iron requirement during surfactant exocytosis. Consistently, disruptions to iron availability, either through extracellular iron chelation or genetic manipulation of MITOIRON transport, significantly impaired surfactant exocytosis. Additionally, these conditions also significantly impaired the wound healing capacity of MLE-12. Interference with MITOIRON had the most

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profound affects across all readouts, simultaneously affecting surfactant exocytosis, synthesis, uptake, and repair.

Chapter 5 built on the dependency of MITOIRON in AT2 cells *in vivo*. Loss of MITOIRON transport in AT2 cells significantly disrupted the cellular ironome, with notable alterations in the expression of iron-binding, heme-associated, and Fe-S cluster-related genes. These molecular disruptions correlated with substantial impairment of AT2 cell function, as evidenced by the loss of AT2 cell markers, accumulation of surfactant proteins in the BALF and reduced maximum surface tension capacity. Importantly, this AT2-specific disruption in MITOIRON metabolism triggered severe lung pathology, characterized by marked inflammation, immune cell infiltration, disrupted iron homeostasis in the lung microenvironment, reduced lung function, and ultimately mortality. This study offers novel and direct evidence linking AT2-specific iron homeostasis to both pulmonary function and pathology.

Surprisingly, AT2 cell iron deficiency correlated strongly with a senescent-like phenotype. Developing on this, **Chapter 6** explored how iron dynamics in AT2 cells contributed to their dysfunction in injury models. *In vitro* AT2 cell iron deficiency precedes iron accumulation and exacerbated cellular senescence findings that were replicated *in vivo* using a murine bleomycin-induced fibrosis model. This thesis uniquely establishes a recurrent signature of AT2 iron deficiency across multiple models (**Chapters 4 – 6**), associating with the initiation of a 'pre-senescence'. Iron depletion appears to trigger early senescence in AT2 cells, subsequently initiating a compensatory iron uptake response that perpetuates cellular senescence (**Chapter 6**). This finding challenges the prevailing dogma that iron accumulation is the primary initiating factor of cellular senescence. Collectively, these findings provide a comprehensive characterization of iron metabolism in AT2 cells, highlighting disrupted MITOIRON metabolism as a potential driver of AT2 cell dysfunction and senescence.

7.1.2 Assembling the Tool-Kit

In **Chapter 3** this thesis enhances the state of the art by offering a comprehensive framework for studying AT2 cell biology *in vitro* and *in vivo*. It addresses a critical gap in the field: the lack of standardized economical methods for assessing the complex functions of AT2 cells, particularly in surfactant production and cellular repair.

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For surfactant, **Chapter 3** highlighted the limitations of focusing on a single aspect of surfactant homeostasis, as surfactant composition, secretion, function, and turnover are distinct yet interconnected elements. The assays developed in this thesis highlight this by adopting a multifaceted approach to investigate each aspect comprehensively. Key considerations were shown when studying surfactant proteins, with this thesis noting that changes in the pro-form might not reflect changes in the mature/secreted form, and similar discrepancies can occur at the transcriptional level.

For repair, a similar approach was taken considering AT2 cell self-renewal, transitional state and AT2 to AT1 differentiation. Specifically, the scratch assay was highlighted for its accessibility. The AT2 to AT1 transition assay, still in development, demonstrated its potential as a valuable tool for examining the transitional capacity of AT2 cells *ex-vivo*. Finally, isolating CD45⁺EPCAM⁺ cell populations from *in vivo* systems - at baseline, during injury, or in AT2-specific gene deletion models - provides insights into AT2 cell repair mechanisms.

By implementing a multifaceted, cost-effective strategy, **Chapter 3** facilitates a detailed overview, supports robust conclusions, and promotes the standardization of methodologies in AT2 cell research.

7.1.2 Powering AT2 Cell Function: Is MITOIRON the Key?

Disruptions in MITOIRON emerged as the antagonist in this thesis due to its detrimental impact on AT2 cell function and overall lung health. Consistently, across existing literature and results presented in **Chapters 4 and 5**, impaired MITOIRON transport disrupts Fe-S cluster and heme synthesis (149-152, 156). These cofactors are essential for multiple facets of energy metabolism, including the TCA cycle (ACO2 and SDH), the ETC (Complex I, II, III and IV) and FAO (ETFDH) (167). Given that AT2 cell surfactant homeostasis and repair processes are energetically demanding, and considering evidence that iron chelation significantly reduces ATP production by interfering with the TCA cycle (278), it is plausible that diminished MITOIRON negatively affects cellular energy output.

Iron is also an essential co-factor for the enzymatic activity of desaturases, which function to introduce double bonds into FA chains to generate PUFAs (349). Studies indicate that rats subjected to iron-deficient diets exhibit marked alterations in lipid metabolism, such as modified cholesterol levels, disrupted cell membrane lipid composition, and impaired synthesis of critical hepatic fatty acids (349). These findings underscore another potential mechanism by which iron deficiency could disrupt surfactant homeostasis. Moreover, the ER, a primary

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site for surfactant lipid synthesis (3), is enriched with iron-dependent proteins (120), suggesting that iron deficiency could directly impair ER function and subsequently indirectly affect surfactant homeostasis.

Notably, observations in **Chapter 4** highlighted how chelation models using DFP and DFX did not impact surfactant homeostasis to the same degree as direct disruptions in MITOIRON transport. Despite intracellular iron reductions, heme levels remained stable in chelation experiments, whereas *Mfrn1/2* deficiency resulted in significantly reduced heme levels, as seen in **Chapters 4 and 5**. This suggests AT2 surfactant homeostasis is particularly sensitive to changes in heme metabolism. While no direct link has been previously established between surfactant homeostasis and heme metabolism, the heme transporter FLVCR1, was recently identified as a plasma membrane choline transporter crucial for choline uptake and metabolism (350). Choline is indispensable for synthesizing PC species used in surfactant production, which AT2 cells cannot produce independently and thus rely on external uptake or recycling (17). Therefore, reduced *Flvcr1a* expression in AT2 cells from RDKO mice may compromise choline availability and, consequently, surfactant synthesis.

Additionally, FLVCR1-mediated choline uptake is integral to maintaining mitochondrial homeostasis (350). Loss of FLVCR1 triggers HRI-mediated ISR activation, presumably linked more closely to impaired heme transport than choline transport (350). Notably, ISR activation is tightly interconnected with mitochondrial dysfunction (179, 351). **Chapters 4 and 5** highlight disruptions in the HRI-ISR pathway. Despite lower iron and heme levels in AT2 cells, resulting from reduced mitochondrial iron transport, *Eif2ak1/Hri* is significantly decreased. However, loss of MITOIRON transport was associated with a trend toward increased expression of *Atf4*, a downstream effector of the ISR (178), suggesting possible ISR activation through alternative kinases.

The ISR facilitates AT2 to AT1 differentiation, with an ISR signature associating with transitional cells in lung disease (70). Alveolar repair studies frequently employ injury models, such as BLM-induced fibrosis, where acute phases are particularly useful for examining epithelial injury and repair (54, 75). **Chapter 6** demonstrated that AT2 cells isolated during the acute injury phase of BLM-induced fibrosis exhibit deficiencies in iron, heme, and reduced *Mfrn1/2* gene expression. However, unlike genetically targeted AT2 cells deficient in *Mfrn1/2* (**Chapters 4 and 5**), these cells retained the ability to upregulate *Eif2ak1/Hri* expression. Single-cell data from the BLM-fibrosis model revealed that during the inflammatory-transitional

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phase, the epithelial population is enriched for transitional cells (KRT8+) with a concurrent reduction in AT2 cells (54, 75). Thus, CD45-EPCAM+ cells isolated at day 8 post-BLM treatment likely represent transitional cells activating the HRI-ISR pathway to facilitate alveolar repair. Conversely, disruptions in mitochondrial iron transport potentially impedes AT2-to-AT1 differentiation, as evidenced by the loss of AT2 markers and accumulation of transitional markers (KRT8+) observed in **Chapter 5**. This suggests that adequate mitochondrial iron transport is essential for proper regulation of the HRI-ISR axis during alveolar epithelial differentiation, linking iron, heme, and ISR pathways to AT2 cell injury and repair for the **first time**.

Additionally, AT2 cells isolated at day 8 from the BLM-induced fibrosis model displayed an iron accumulation gene signature, characterized by increased expression of iron uptake and transport genes. Similarly, single-cell analyses highlight LCN2 as significantly upregulated in activated/transitional AT2 cells following BLM injury (75). LCN2 mitigates tissue damage by sequestering excess iron, protecting epithelial integrity (7). Alongside other iron-binding proteins identified in BALF, LCN2 may serve to buffer epithelial cells against iron-mediated toxicity (183-185).

Furthermore, recent research demonstrates that age-related iron deficiency significantly compromises the reparative and regenerative capacities of AT2 cells, implicating the NUPR1-LCN2 axis (267). However, since LCN2 is secreted, activation of the NUPR1-LCN2 axis offers limited direct insight into intracellular iron concentrations. This thesis represents **the first** characterization of the iron status of AT2 cells within an injury context. With both the literature and this thesis consistently pointing to iron deficiency as a factor in AT2 cell injury, repair and dysfunction, we hypothesized that BLM induces its fibrotic effects by causing iron deficiency in AT2 cells, disrupting pulmonary iron homeostasis. In response, AT2 cells upregulate iron-binding proteins to sequester alveolar iron, in an attempt to mitigate iron-induced damage

7.1.3 AT2 Cells and Iron Regulation in the Lung

Recent research has increasingly focused on the mechanisms of lung-iron regulation, given its critical role in the pathogenesis of various lung diseases (182). Under normal conditions, lung iron is governed by the IRP-IRE axis (188, 189). In lung disease, this system can become dysregulated leading to inappropriate iron storage or release within lung cells, affecting their function and contributing to disease pathology (182).

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To date there has been no cell-specific approach when exploring lung-iron regulation with the majority of research employing disruptions in iron systemically or intratracheal instillation of siRNA vectors (188, 189, 194, 267). The research from **Chapter 4 and 5** is the first of its kind to target iron metabolism in any specialized cell in the lung. Utilizing the AT2-specific gene deletion system *in vivo*, we demonstrated that impaired MITOIRON transport in AT2 cells leads to increased levels of iron, heme, and porphyrins in the BALF. Additionally, whole lung-iron decreases, without compensatory HRI protein upregulation. Despite significant increases in TFRC and FTH1, these regulatory proteins fail to normalize lung-iron levels, suggesting a loss in the IRP-IRE axis. Evidence also shows a disruption of the IRP-IRE axis in *Mfrn2^{CRISPRi}* cells, where FTH1 levels remain unchanged despite significantly reduced iron levels. This underscores the critical role of mitochondrial iron transport in both AT2 cell iron sensing and overall lung iron regulation.

Moreover, inflammation and stress responses propagated by AT2 cells may also influence lung iron levels. A deficiency of *Mfrn1/2* in AT2 cells leads to inflammation, as shown by elevated IL-6 and TNF- α levels in BALF and lung tissue. Additionally, in the lung tissue of RDKO mice, increased expression of p53 and p21 proteins suggests activation of the cell-cycle arrest pathway. Given the known associations between both inflammatory (352) and cell-cycle arrest pathways (281) with iron metabolism, it is plausible that these pathways are affecting iron regulation in the lung.

7.2 Future Directions

7.2.1 Unresolved Questions

A key unresolved question in this thesis concerns the effects of iron deficiency on mitochondrial function in AT2 cells. While the critical role of iron in mitochondrial metabolism is well established (167), direct examination of mitochondrial function was not carried out in this thesis. Using Seahorse technology could shed light on how changes in iron availability and disruptions in the mitochondrial iron transport system affect mitochondrial respiration. Additionally, further insights could be gained by isolating mitoplasts and probing for proteins involved in OXPHOS and FAO. Ideally, these techniques would be applied to AT2 cells isolated from RDKO mice. However, the practical challenges associated with culturing AT2 cells *ex vivo* and obtaining mitoplasts from primary cells, due to low yields, make these approaches particularly challenging. Alternatively, these techniques could be applied to the *in vitro* systems described in **Chapter 4**.

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This thesis proposes a **novel role** for mitoferrin 1 and 2 as crucial regulators of the HRI-ISR in AT2 cells, but substantial gaps remain before drawing definitive conclusions. A critical area of future focus would be the investigation of phosphorylated-HRI, the activated form of the kinase. Additionally, future research should extend to other ISR-activating kinases and downstream effectors to assess whether the pathway is fully compromised in AT2 cells with impaired mitochondrial iron transport. Of note, the HRI-ISR axis can be activated by mitochondrial dysfunction (351), which in turn can initiate the process of mitophagy (180). Given the strong link between iron metabolism and mitochondrial health in AT2 cells, it is particularly relevant to explore mitochondrial dynamics in this context. Specifically, investigating whether impaired MITOIRON transport and compromised HRI-ISR activation influence mitophagy in AT2 cells could provide valuable insights.

Investigating the contributions of the HRI-ISR axis and mitochondrial metabolism to alveolar repair is essential, as both have been linked to AT2 mitochondrial iron transport in this thesis. By characterizing MITOIRON dynamics during normal AT2-to-AT1 transdifferentiation and comparing this with an impaired HRI-ISR response could provide deeper insights into how MITOIRON is harnessed to support repair processes.

The differences between HetDKO and RDKO mice raise questions about why HetDKO mice maintain health at baseline, hinting at potential alternative mitochondrial iron transporters as discussed in **Chapter 5**. Investigating whether reintroducing iron via dietary or intravenous supplementation in RDKO mice could rescue AT2 cells, would provide insights into these alternative transport mechanisms. Additionally, employing CRISPR/Cas9 screens could precisely identify alternative mitochondrial iron transport pathways in AT2 cells. Notably, our 'Iron Hungry' model (**see Chapter 6, section 6.2.6**) suggests that iron deficiency might irreversibly impair iron regulatory functions in these cells, evidenced by persistent and worsening p21 expression even after iron supplementation. Significant differences in iron handling between *in vitro* systems, which can experience iron overload (334), and *in vivo* environments, where iron is regulated by iron-binding proteins, underscore the necessity of extending investigations to both *in vitro* and *in vivo* models.

An additional outstanding question is the mechanism by which AT2 cells lose iron in response to injury. One potential mechanism involves FPN; notably, despite cellular iron deficiency observed in early acute injury models, transcriptional upregulation of FPN is markedly increased (**Chapter 6**). Given their distinctive endocytic capacity, it is also plausible that AT2

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cells may deplete their intracellular iron stores through the endocytosis and subsequent shedding of extracellular vesicles enriched in iron-containing compounds (e.g. ferritin).

The underlying rationale for this iron loss remains unclear: does it represent an active cellular response to injury, or is it merely a passive consequence of exposure to injurious stimuli? A key limitation of the BLM model in this context is its inherent interaction with iron metabolism, particularly its underappreciated role as an iron chelator (340-344). This complicates the assessment of iron dyshomeostasis in AT2 cells, as it might merely reflect a BLM specific response. *In vitro* studies exploring the effects of CSE also encounter challenges, with particulate matter in CS thought to complex with host iron (353). These observations represent a mere snapshot in time. Therefore, extending investigations into the recovery resolution phase and utilizing additional injury models may provide insight into how shifts in iron metabolism facilitate AT2 cell repair over time.

Finally to fully grasp the broader implications of AT2-specific iron dysregulation further characterization of the severe effects observed in RDKO mice is essential. Specifically, given the increased infiltration of neutrophils and AMs highlights the potential of flow cytometric analysis on whole lung tissue from RDKO mice to provide deeper mechanistic insights into the immune cell populations accumulating in the lung. Preliminary *in vitro* CM experiments have already linked AT2 cell iron metabolism to AM function, demonstrating that AT2 cell iron regulates not only its own function but may also indirectly influence neighbouring cells. Employing advanced cell culture techniques such as air-liquid interface models or co-culture organoid models could be utilized to understand this relationship further. Additionally, given the disruptions observed in FGF signalling, co-culture of AT2 cells with fibroblasts and/or isolation of fibroblast from RDKO mice could provide further mechanistic insights into how MITOIRON dysregulation in AT2 cells disrupts this signalling pathway.

Finally, given the dramatic disruptions observed in lung iron, it would also be valuable to examine whether these effects manifest at a systemic level. Specifically, exploring whether the systemic FPN-hepcidin axis is activated in response to these changes would be particularly informative. This could help determine if lung-specific iron dysregulation can influence systemic iron homeostasis.

7.2.2 Tool Limitations

A significant limitation of this thesis is the predominant reliance on mRNA profiles to infer underlying mechanisms. Given that protein regulation is influenced by post-translational modifications, degradation, and substrate availability, mRNA expression may not accurately reflect protein levels (354). Consistent with this, discrepancies between *SftpC* transcript levels and both pro and mature protein forms were observed, underscoring the necessity to apply a dual approach of both mRNA and protein analyses. Techniques such as mass spectrometry-based proteomics could offer more detailed insights underlying mechanisms (355).

Further development of specialized tools to evaluate AT2 cells' differentiation into AT1 cells, as well as their self-renewal and transitional states, will offer a comprehensive understanding of AT2 cell repair capacity. Organoid cultures can facilitate studies on AT2 cell self-renewal, while transdifferentiation assays provide insights into the conversion of AT2 to AT1 cells and transitional cell dynamics. Additionally, employing flow cytometry will allow for the analysis of various AT2-like cell populations across different models, enhancing our understanding of these cellular processes. Furthermore, given the binary nature of these assays, it is crucial to concurrently explore signalling pathways.

AT2 cells in the lung constitute a heterogeneous population with distinct functions: maintaining the AT2-cell stem niche, differentiating into AT1 cells, or facilitating surfactant secretion (54, 66, 75, 356). However, the standard method of isolating AT2 cells using EPCAM⁺CD45⁻ selection does not distinguish among these functional subtypes. Moreover, during the enrichment of primary AT2 cells, subsets that express both club and AT2 cell markers are inadvertently excluded. These cells are capable of differentiating into AT2 cells under specific conditions (100), resulting in the loss of potentially significant biological signals and influences during studies of AT2 cell repair and renewal.

Additionally, in lung disease models, distinct AT2 cell subsets have been identified (95, 99, 100, 357), yet here we also applied a uniform approach. It is possible that only a subset of AT2 cells is particularly vulnerable to iron deficiency-induced damage, akin to the subset of AMs in IPF that are more susceptible to iron loading (207). This highlights the heterogeneous responses of the same cell type to dysfunction. Similarly, senescence is a heterogeneous process, with only a fraction of cells undergoing senescence (107). Investigating whether AT2-specific iron dysregulation aligns with specific AT2 subsets would be of interest.

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Finally, an overarching limitation of this thesis is the reliance on murine models both *in vitro* and *in vivo* to investigate AT2 cell function in both health and disease. There are several fundamental differences between mice and humans particularly when it comes to the lungs. Specifically, structurally human lungs feature a gradual transition from proximal airways to alveolar ducts, populated by a unique secretory cell type, RAS cells, located in the respiratory bronchioles (358). These RAS cells under specific conditions can differentiate into AT2 cells. In contrast, murine lungs lack these detailed transitional zones and specific cell types (358). Therefore, caution must be exercised when interpreting these results, and findings from this thesis require validation through studies in human tissue or clinical data.

7.3 Translational Aspects

With the expanding research into AT2 cell biology, this thesis enhances the understanding of how iron metabolism influences AT2 cell function. Given the critical role of AT2 cells in various lung diseases, identifying molecular cues and nutrients that modulate their function is essential for both basic biology and the development of targeted therapeutics. Thus, the findings presented here provide foundational insights for the therapeutic targeting of iron metabolism in AT2 cells.

Iron chelators have recently emerged as potential therapeutic agents in chronic lung diseases, with *in vivo* studies indicating their efficacy in managing lung iron overload (118, 208, 359). For example, DFX administered in combination with BLM in experimental models of fibrosis effectively reduced pulmonary iron deposition (208). Similarly, prophylactic use of DFP in CS-induced experimental models of COPD alleviated bronchitis and emphysematous pathology (118). Of note, these iron chelators were delivered systemically via drinking water (DFP) or intravenously (DFX), prompting suggestions for more targeted pulmonary delivery (118). The present thesis, however, underscores the potential detrimental effects of iron depletion in AT2 cells, guiding future therapeutic strategies and emphasizing caution in utilizing iron chelators directly in the lung, suggesting a more cell-specific targeted approach to ameliorate iron-loaded cells in the lungs like AMs.

Senolytics, drugs targeting senescent cells, have shown promise for the treatment of conditions associated with accelerated cellular aging (360). Given their senescent phenotype, AT2 cells represent a primary therapeutic target for senolytic interventions. Early clinical trials using dasatinib plus quercetin, demonstrated improvements in physical function among

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individuals with IPF (361). However, senolytics often lack cellular specificity, raising concerns regarding off-target effects on 'healthy' aged cells. To address this, recent research has explored iron-activated senolytic prodrugs, exploiting the tight link between iron accumulation and cellular senescence, offering enhanced specificity towards senescent cells (107).

Despite promising *in vitro* results, translating this strategy to *in vivo* lung systems presents potential risks. The targeted elimination of iron-rich senescent cells could release substantial amounts of free iron (107), overwhelming local iron-binding proteins and exacerbating lung injury (119, 184, 185). Moreover, this thesis highlights the detrimental consequences of excessive iron exposure, particularly in the context of AT2 cells with dysregulated iron metabolism. Given that AT2 cells in IPF lungs appear iron-deficient according to the IPF cell atlas (**Figure 6.18**), therapeutic strategies that inadvertently elevate local iron concentrations could paradoxically exacerbate disease pathology. One potential therapeutic strategy could involve the co-delivery of iron-activated senolytic prodrugs with DFP. Preliminary *in vitro* experiments presented in **Chapter 4** demonstrated the efficacy of DFP in MLE-12 cells, where treatment did not induce senescence or SASP markers, and changes in IRE transcripts were minimal. Notably, DFP enhanced wound healing capacity, suggesting a potential role in promoting epithelial repair. Given DFP's unique ability to shuttle iron between cellular compartments (118, 272), it may modulate MITOIRON levels, preventing both iron overload and deficiency. As such, combining senolytics with DFP could enable targeted iron chelation and redistribution, addressing extracellular iron accumulation while preserving intracellular iron availability in AT2 cells.

7.4 Concluding Remarks

In conclusion, this thesis significantly advances our understanding of iron biology by uncovering a novel and critical role for MITOIRON metabolism in the specialized functions and integrity of AT2 cells. Through an integrative combination of *in vitro* and *in vivo* models, this research has demonstrated for the first time how precise manipulation of mitochondrial iron transport profoundly impacts surfactant homeostasis, alveolar repair processes, and overall lung health.

Importantly, this work revealed a previously unappreciated paradoxical relationship between iron deficiency and cellular senescence within AT2 cells, challenging current paradigms that primarily associate iron overload with senescence. The novel observation that MITOIRON depletion in AT2 cells predisposes these cells toward a senescent-like state and subsequent iron accumulation provides critical insights into the underlying iron-disarray associated with lung disease.

Ultimately, the findings presented in this thesis represent a substantial leap forward in the field of AT2 cell biology, positioning mitochondrial iron metabolism as a central factor in maintaining cellular function. By elucidating the intricate link between MITOIRON balance, surfactant metabolism, and cellular senescence, this work lays the foundation for novel therapeutic approaches aimed at restoring mitochondrial iron homeostasis to combat lung diseases characterized by compromised AT2 cell function and senescence.

8. Published Work

8.1 Published Papers

1. **Kenny S***, Faherty L*, and Suzanne M. Cloonan, Iron and mitochondria in the susceptibility, pathogenesis and progression of COPD. *Clinical Science*, **2023**. 137(3): p. 219-237.
2. **Kennys S***, Schweikert A*, Oglesby I, Glasgow A, De Santi C, Gensch I, Lachmann N, Desroziers T, Fletcher C, Snijders D, Nathan N, Hurley K: **2023** Patient-Derived iPSC Models of Interstitial Lung Diseases: An Evaluation of the Open-ILD COST Training School.
3. Fan, LC., Mc Conn, K., Plataki, M., **Kenny, S.**, Williams, N., Kim, K., Quirke, JA., Sauler, M., Möbius, ME., Chung, KP., Gomez, EA., Choi, A., Xu, JF., Cloonan, SM: **2024**. Alveolar type II epithelial cell FASN maintains lipid homeostasis in experimental COPD.
4. Healy, C., Munoz-Wolf, N., Strydom, J., Faherty, L., Williams, N., **Kenny, S.**, Donnelly, S., and Cloonan, S. **2021**. Nutritional Immunity: The impact of Metals on Lung Immune Cells and the Airway Microbiome during Chronic Respiratory Disease

8.2 Conference Abstracts

1. **Kenny, S.**, Huang, Z., Cloonan, C. Type II alveolar epithelial cell dysfunction associates with altered iron levels after bleomycin treatment. **Poster** session and **presentation** presented at: Irish Thoracic Society Meeting. Online. November 2021
2. **Kenny, S.**, Huang, Z., Kiwhan, K., Capili, A., Carpenter, S., Ward, D., Cloonan, S., Mitochondrial iron regulates AEC2 dysfunction, senescence and senescent associated secretory phenotype in response to bleomycin challenge: **Poster** presentation at ERS Lung Science Conference. Estoril, Portugal. March 2022
3. **Kenny, S.**, Huang, Z., Kiwhan, K., Capili, A., Carpenter, S., Ward, D., Cloonan, S., Iron dyshomeostasis and alveolar type II cell dysfunction in idiopathic pulmonary fibrosis. **Poster** presentation at the European Iron Conference. Oxford, UK. June 2022
***Awarded young researcher travel bursary award*
4. **Kenny, S.**, Huang, Z., Kiwhan, K., Capili, A., Carpenter, S., Ward, D., Cloonan, S., Iron dyshomeostasis and alveolar type II cell dysfunction in idiopathic pulmonary fibrosis: **Poster** presentation at International Colloquium on Lung and Airway Fibrosis. Reykjavík, Iceland. October 2022.
5. **Kenny, S.**, Huang, Z., Kiwhan, K., Capili, A., Carpenter, S., Ward, D., Cloonan, S., Ironing out the role iron plays in alveolar type II epithelial cell function **Poster**

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presentation at the Pittsburg International Lung Conference. Dublin, Ireland. October 2023.

***Awarded best poster presentation*

6. **Kenny, S.**, Huang, Z., Kiwhan, K., Capili, A., Carpenter, S., Ward, D., Cloonan, S., Exploring iron dynamics in alveolar type II epithelial cells: Implications in chronic lung diseases. **Poster** presentation at the European Iron Club. April 2024.
7. **Kenny, S.**, Huang, Z., Kiwhan, K., Capili, A., Carpenter, S., Ward, D., Cloonan, S., Mitochondrial iron dysregulation in alveolar type II epithelial cells: Implications for idiopathic pulmonary fibrosis. **Poster** and **Oral** presentation at the young investigators meeting, CA21115 – FeSImmChemNet. Berlin, Germany. September **2024**.
8. **Kenny, S***, Huang, Z*, Kiwhan, K., Kihwan, K., Gesmundo, S., Palataki, M., Ward, D., Cloonan, S., Targeted loss of MITOIRON in the alveolar epithelium promotes lung injury and fibrosis. **Poster** presentation and **Flash-Talk** at the International BIOIRON Conference. Montreal, Canada. May 2025

***Awarded best oral and poster presentation*

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