ALVEOLAR TYPE 1 EPITHELIAL CELL DEFICIENCY IN PULMONARY HYPERTENSION

by

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Abstract

Rationale: Pulmonary Arterial Hypertension (PAH) is a severe health condition that involves an ongoing process of pulmonary vascular resistance and vascular remodeling. These changes can eventually cause right heart failure (RHF) and in severe cases and can eventually result in death. AT1 cells mediate gas exchange in the lung. However, the connection between AT1 cell dysfunction and the onset of pulmonary hypertension (PH) is still not well-understood. A better understanding of the mechanisms could help the improvement of treatment for PAH.
Objectives: To investigate the role and underlying mechanisms of AT1 cell deficiency in the pathogenesis of PAH.
Methods: To determine whether AT1 cells are deficient in the development of PAH, lung sections from idiopathic PAH (IPAH) and control donors, as well as lung tissues from PH mice model Egln1Tie2cre (CKO) mice and wild-type (WT) mice, were evaluated for the expression of AT1 cellmarkers. Reverse Transcription-quantitative PCR (RT-qPCR) was performed in mice samples, to determine the expression levels of AT1 cells markers (Ager, Aqp5, Hopx, Rtkn2) using lung tissues from CKO and WT mice. Western blot analysis was then performed to quantitatively evaluate AT1 cell marker protein levels (Ager, Hopx) in lungs from CKO and WT mice. Immunofluorescence staining was applied using HOPX in both mice lungs and human IPAH lungs. Additionally, the expression of VEGFA, AT1 derived factor, was determined using the RNAscope technique on human IPAH and control lungs.
Results: The findings of this study showed the mRNA levels of AT1 markers including Hopx and Rtkn2 were downregulated in CKO mice. The expression levels of AT1 markers Ager but not Hopx was downregulated in CKO mice by Western Blot. A decrease of AT1 cells assessed by quantification of HOPX in the IPAH human samples and CKO mice was observed in Immunofluorescent staining. Lastly, upregulated VEGFA expression in IPAH human samples were recorded as revealed by the RNA scope.
Conclusion: The findings of this study demonstrated that there is a deficiency of AT1 cells in PAH patients and PH mice. Deficient AT1 cells might lead to impaired regeneration of the pulmonary vasculature and the development of PAH. Our study might provide a novel concept to treat PAH patient via studying the AT1 cells and their underlying signaling.

Keywords

Endothelial cells	Pulmonary hypertension	RNASCOPE
AT1 cells	Antibody	CKO
AT2 cells	Western Blot	WT
Egln1tie2cre	Quantitative RT-PCR	hypoxia
VEGFA	Immunofluorescence staining	IPAH

Abbreviations

(PH) Pulmonary Hypertension
(RHF) Right Heart Failure
(AT1 cells) Alveolar type 1 epithelial cells
(AT2 cells) Alveolar type 2 epithelial cells
(EC) Endothelial cells
(WT) Wild type
(CKO) Conditional knockout

(PVECs) Pulmonary Vascular Endothelial Cells
(IPAH) Idiopathic Pulmonary Arterial Hypertension
(VEGFA) Vascular Endothelial Growth Factor A
(RT-qPCR) Reverse Transcription-Quantitative Polymerase Chain Reaction
(HOPX) Homeodomain Only Protein X
(RAGE) Receptor for Advanced Glycation End Products
(AQP5) Aquaporin 5
(RTKN2) Rhotekin 2
(SP-C) Surfactant Protein C
(PDPN) Podoplanin
(IHC) Immunohistochemistry
(WHO) World Health Organization
(COPD) Chronic obstructive pulmonary disease
(CH) Chronic Hypoxia
(CTEPH) Chronic Thromboembolic Pulmonary Hypertension
(PTE) Pulmonary Thromboendarterectomy
(HIF-2α) Hypoxia-inducible factor 2-alpha
(PHBI) Pulmonary Hypertension Breakthrough Initiative
(CT) Cycle threshold

Introduction

Pulmonary Hypertension (PH) is a serious medical condition characterized by high blood pressure in the arteries that carry blood from the heart to the lungs. This ongoing increase in pressure is linked to the narrowing and remodeling of these arteries, which puts more strain on the heart as it pumps blood through the lungs. This may eventually result in cardiac failure and premature death. [1] The cause of PH is frequently complicated and poorly understood. Genetic predisposition, certain medications or chemicals, and underlying illnesses such as connective tissue disorders or left heart disease are known risk factors.

Groups of PH

There are 5 groups of PH classified by the World Health Organization (WHO).

Group 1 of the WHO classification pertains to a condition known as **Pulmonary Arterial Hypertension (PAH)**. PAH manifests when when resitance develops in the pulmonary hypertension due to enhanced vasoconstriction, vessel stiffening,. This results in the right side of the heart straining more to pump blood through these narrowed arteries. This added exertion can lead to an impairment in the heart's capacity to circulate sufficient blood throughout the lungs to meet the demands of the body. [2]

The WHO Group 2 classification encompasses PH that arises from left heart disease. In this category, the arteries are not as stiff or inflexible as those seen in WHO Group 1, but there are issues with the heart's contraction or relaxation functions, or malfunctions with the heart valves on the left side. These complications prevent the left heart from efficiently processing the incoming blood from the lungs, leading to a "backlog" of blood that increases the pressure within the lungs. This WHO Group 2 category represents the most commonly observed form of PH. [2]

Group 3 pertains to PH resulting from chronic lung diseases and/or hypoxia, a state of low oxygen levels. This encompasses obstructive lung conditions that cause narrowing of lung airways and difficulty exhaling, such as COPD or emphysema; restrictive lung diseases that make lung expansion challenging during inhalation, like interstitial lung disease or pulmonary fibrosis; sleep apnea; and prolonged residence in high-altitude regions. In these cases, the pulmonary arteries constrict to direct blood only to those parts of the lungs receiving maximum air and oxygen. This constriction triggers a rise in blood pressure across the lungs. Chronic Hypoxia (CH) leads to alterations in the pulmonary circulation, causing PH and right ventricle enlargement (RV hypertrophy), ultimately risking right heart failure. In those affected by CH, the onset of PH is linked with substantial morbidity and mortality. Nevertheless, the options for treating this condition are limited, and the methods for prevention remain largely undiscovered or undefined. [3]

Group 4 refers to Chronic Thromboembolic Pulmonary Hypertension (CTEPH). CTEPH can develop when the body is unable to break down a blood clot in the lungs, leading to scar tissue formation in the lung's blood vessels. This obstruction hampers regular blood flow, causing the right side of the heart to exert extra effort. This form of PH stands out as it can potentially be resolved through a surgery called Pulmonary Thromboendarterectomy (PTE) to remove the blood clots. However, this surgical option isn't suitable for all CTEPH patients. A specific medication is also available for CTEPH patients if they are deemed unsuitable for the PTE surgery or if PH persists post-surgery. [2]

Lastly, Group 5 classification denotes cases where PH is a secondary outcome of other diseases, through mechanisms that are not yet fully comprehended. The conditions linked with this form of PH are numerous and include, but are not confined to, diseases such as sarcoidosis, sickle cell anemia, chronic hemolytic anemia, splenectomy (removal of the spleen). [2]

PAH Mechanisms: Focus on Endothelial Signaling

Our understanding of PAH mechanisms has significantly evolved over time. It's known to involve complex interactions between the monolayer of cells lining the blood vessels, called the endothelial cells (ECs) and muscular underlying layer, the smooth muscle cells (SMCs). One previous treatment approach was tested and provided illuminating results. Endothelial HIF-2a signaling was characterized as a novel therapeutic target for the PH26 gene. Research also characterized the novel pathways through which endothelial autocrine and paracrine signaling mediated pulmonary vascular remodeling and PH development. It became evident that EC injury or dysfunction was the initial event during the onset of PH development. Identification of how EC injury or dysfunction occurred potentially led to an improved scientific understanding of PAH etiology and treatment.

The endothelial cells are especially crucial in this process as they regulate vascular tone, inflammation, and blood clotting. When endothelial dysfunction occurs, due to hypoxia this results in an imbalance in vasodilators (like nitric oxide) and vasoconstrictors (like endothelin) leading to increase pulmonary resistance, inflammation, where chronic inflammation can lead to remodeling of the vascular wall, and thrombosis, in which formation of blood clots within the pulmonary vessels can increase resistance and pressure. Genetic factors such as mutations affecting the BMPR2 gene are associated with PH as well as underlying diseases, for example, Liver Disease, Connective tissue Disease, and Chronic Lung Disease. Left Ventricular Dysfunction and Mitral Valve Disease can also cause a back-up of blood into the pulmonary

circulation, creating elevated pressure. Treatments can be complicated and typically must be individualized based on the underlying cause and mechanism; in severe cases, this may include anticoagulation, lung transplantation, or vasodilator therapy. [3]

Despite improvements in our understanding, there are still many undiscovered elements of PAH. The genetic, molecular, and cellular elements of PAH require further study, particularly the role of alveolar lung epithelial cells, a subject that has received very little attention and research.

Type 1 Alveolar Epithelial (AT1) Cells

Alveolar type 1 (AT1) cells are epithelial cells that are located on the alveoli, the small lung pouches essential for breathing. They are vital for gas exchange as they play a role in transferring oxygen (O_2) from the air we breathe to our blood and removing carbon dioxide (CO_2) from the bloodstream. In terms of structure, AT1 cells are notably expansive and slim, making up almost 95% of the surface within the alveoli. Such attributes enhance the movement of gases. As oxygen travels from inhaled air, it moves through AT1 cells, then through a fine basement membrane, before passing through the lung's blood vessel walls and finally entering our blood's red cells. On the other hand, CO_2, a byproduct from our body's cells, takes the reverse route to be eventually exhaled. Beyond gas transfer, AT1 cells are crucial for maintaining the alveoli's shape and structure. They establish firm connections with adjacent cells, crafting a protective shield separating the breathable air from the fluid in the surrounding tissues. They also help in averting the potential sticking and consequent collapse of alveoli, which could result from the liquid lining on the alveoli surface. While alveolar type 2 cells primarily produce the surfactant that reduces this surface tension, AT1 cells ensure it spreads effectively, supporting lung stability and making breathing smoother. In essence, AT1 cells, with their distinct structure and roles, are key to effective gas exchange and the structural integrity of the lungs. AT1 cells feature certain types of markers and antibodies that are designed to identify and locate AT1 cells and their expression in different experiments. Homeodomain-only Protein X (HOPX) is an important transcriptional regulator, and in the context of the lung, it plays a role in cell differentiation, ensuring the correct development and function of AT1 cells, which are vital for efficient gas exchange in the alveoli. Podoplanin (PDPN) is a mucin-type protein expressed on the surface of AT1 cells. It has been recognized as a reliable marker for distinguishing them from other lung cells, especially AT2 cells. Receptor for Advanced Glycation End Products (RAGE) is a multi-ligand receptor expressed predominantly on the surface of AT1 cells in the lung. It has been linked to various cellular processes, including inflammation and tissue homeostasis, and its expression in AT1 cells aids in their identification. Rhotekin 2 (RTKN2) is an AT1 marker protein involved in actin cytoskeleton organization and cell morphogenesis. Aquaporin 5 (AQP5), a water channel protein, plays a significant role in the movement of water across cell membranes. In the lung, AQP5 is localized predominantly to AT1 cells, facilitating water transport essential for maintaining the thinness of the air-blood barrier, which is critical for effective gas exchange. Collectively, these markers provide tools for the specific identification and study of AT1 cells in the lung in PAH.

Type 2 Alveolar Epithelial (AT2) Cells

Alveolar Type 2 (AT2) cells are specialized epithelial cells in the lungs that have several important functions. They are known primarily for their role in producing and secreting pulmonary surfactant, a lipid-protein complex that reduces the surface tension in the alveoli

and prevents their collapse during expiration. In addition, AT2 cells serve as progenitor cells for the alveolar epithelium, meaning they can differentiate into (AT1) cells for alveolar repair and regeneration. One AT2 cell marker commonly known as surfactant protein C (SP-C) is an essential part of lung surfactant.[5,6] A typical method for identifying the prevalence and dispersion of SP-C in lung tissue samples is immunohistochemistry (IHC) labeling.[7,8]

Endothelial Cells (ECs)

ECs are a vital component of the circulatory system, lining the interior surface of blood and lymphatic vessels, from the smallest capillaries to the largest arteries and veins. As a monolayer, these cells serve as a selectively permeable barrier between the vessel lumen and the surrounding tissue, regulating the exchange of materials such as oxygen, nutrients, hormones, and waste products. Additionally, they play pivotal roles in vasodilation and vasoconstriction by producing nitric oxide and endothelin, respectively, modulating blood flow and blood pressure. Endothelial cells also line the pulmonary arteries in the lungs. In addition to various cell types already identified to play a part in the development of PH, such as smooth muscle cells, fibroblasts, and leukocytes, recent research highlights a vital role for endothelial cells (ECs) in both the onset and progression of PH. The involvement of ECs in PH is complex and influences a range of pathophysiological processes. These include constriction of blood vessels, inflammation, blood clotting, metabolism, and oxidative/nitrative stress, as well as aspects of cell survival, proliferation, and differentiation. [9]

AT1 and ECs Link

EC's are known to regulate vascular functions such as blood flow and coagulation. The link between AT1 cells and ECs is defined by their close proximity and mutual function in gas exchange. Within the alveoli, AT1 cells and ECs are adjacently placed, with only a slender basement membrane separating them. This arrangement facilitates the swift transfer of oxygen from the alveoli through the ECs into the blood and the simultaneous removal of carbon dioxide in the reverse direction. The interaction and proximity of AT1 cells and ECs are crucial for the optimal operation of the respiratory system, ensuring efficient oxygen delivery to the blood and effective carbon dioxide removal. The coordinated effort of these cellular groups is essential to sustaining lung health.[9] In terms of PH, the growth factor known as Vascular Endothelial Growth Factor A (VEGFA), is mainly expressed by AT1 cells. VEGFA is a crucial protein primarily responsible for orchestrating angiogenesis which is the formation of new blood vessels from existing ones. This protein is important in several physiological processes, including embryonic development, wound healing, and the menstrual cycle. [10]

Hypothesis

The role of AT1 cell deficiency in PAH is a subject of investigation with very little substantial research. Previous studies demonstrated that human lung tissue and $Rosa^{mTmG}$ CKO mice exhibited a downregulation in AT1 cells. In comparison to normal control lungs or nearby unaffected AT1 cells within the same lung, the targeted AT1 cells displayed an upregulated expression of HOPX. In addition, they also found the downregulation of other AT1 cell markers like Rage and an increase in the proliferation indicator KI67. [10] Therefore, we hypothesize that using the combination of AT1 markers, such as HOPX, Rage, Aqp5, and Rtkn2, will demonstrate a decrease of AT1 cells and cell expression within human and $Egln1^{tie2cre}$ CKO

mice lung tissues. To demonstrate this hypothesis, a series of experiments to detect AT1 expression within the PH effected lungs are performed.

Materials and Methods

Human Samples

A study that examined 3,381 individuals indicates that approximately 2.6% of the general population shows potential echocardiographic indicators of PH. Hereditary and IPAH which were once called primary PH, are types of PH. The University of Arizona's Institutional Review Board gave approval for the use of stored human lung tissues and cells. Pulmonary Vascular Endothelial Cells (PVECs) and lung tissues from patients with IPAH and non-usable donors were sourced from the Pulmonary Hypertension Breakthrough Initiative (PHBI).

Mice Models

$Egln1^{Tie2}$ mice (CKO) from Jackson's lab were generated by breeding Egln1 floxed (WT) mice with mice containing the Tie2 promoter/enhancer driven Cre transgene. The study included both male and female mice and followed the National Institutes of Health guidelines for using laboratory animals. The University of Arizona Institutional Animal Care and Use Committee approved the animal care and study protocol.

Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) analysis

One of the approaches undertaken to explore the genetic contributors to PH involves quantitative polymerase chain reaction (qPCR), a potent method used to amplify and simultaneously quantify a targeted DNA molecule, enabling the detailed study of gene expression. This method offers an opportunity for in-depth study of gene expression patterns and their roles in health and disease.

Known for its ability to identify and measure target DNA sequences, qPCR provides the opportunity to assess the levels of gene expression under various environmental conditions. In the current study, gene expression in WT and samples with CKO mice is compared. Such comparisons provide a much more thorough knowledge of the disease's molecular landscape by shedding light on the genes and pathways changed in PH.

In the context of this experiment, precisely designed primers were used in qPCR to assess the expression of five different genes. Pre-existing comprehension of the primers' significance in PH is one of many elements that affect them as the starting point for DNA strand replication. The findings of this study will provide insight into the unique role played by each gene in the development of PH. [11,12,13]

The acquisition of all required materials served as the first step in the qPCR experiment's setup. 4 primers were designed and ordered using NCBI primer-BLAST, by investigating the gene and type of DNA to be tested along with a dilution of Cyclophilin. Ager (Rage), Aqp5, Hopx, Rtkn2, these AT1 cell markers were the specific targets for the primers. The

Cyclophilin primer was used as the control. A total of ten cDNA mice samples, five of WT and five CKO were tested. [14]

The PCR plate, with each sample of WT and CKO consisting of 10 ng cDNA, was carefully inserted into the qPCR machine when sample loading was complete. The software was set to the preferred denaturation, annealing, and elongation thermal cycling settings for the amplification process, which normally involved several cycles of denaturation, annealing, and elongation. The machine was further configured to measure the amount of amplified DNA by measuring the fluorescence dye, known as SYBR-green, after each cycle. Real-time data was collected and evaluated throughout the qPCR cycle. Following calibration against the Cyclophilin control, the relative expression of the target genes Ager, Aqp5, Hopx, and Rtkn2 in the WT and CKO samples were used to determine the final outcomes. [15,16,17] Following the qPCR run, a thorough comparison of the gene expression levels between the WT and CKO samples was carried out, with an emphasis on the significance of these results for the related biological processes.

Western Blotting

Western blot, an indomitable analytical technique in molecular biology and genetics, has been extensively used in scientific research to detect specific proteins in a sample of tissue homogenate or extract. This study focuses on the application of western blotting using two antibodies for AT1 cell markers: Hopx (E-1 Homeodomain-Only Protein X, SC-398703, lot # D1522 mouse monoclonal Santa Cruz Biotechnology) and Ager (Rage) (Receptor for Advanced Glycation End Products, SC-80652 mouse monoclonal, Santa Cruz Biotechnology). Hopx is the AT1 cell biomarker associated with various biological processes, including cell differentiation and organ development. [18,19] It has gained significant attention due to its potential involvement in numerous pathologies and specifically PH. On the other hand, the Receptor for Advanced Glycation End Products or Ager (Rage), is also an AT1 cell biomarker, as mentioned in the previous q-PCR, and a multiligand cell surface receptor that plays a crucial role in inflammation, cell migration, proliferation, and survival. Beta actin was used as a loading control. [20,21] This setup was designed to accommodate the 6 CKO and 6 WT mice samples alongside a molecular weight marker. The 15% SDS-PAGE gel was made for the western blot and was run at a constant current at 25V and then transferred to a membrane. The transferred membrane was incubated in the automatic western blot processing machine with the primary antibodies for Hopx, Rage, diluted at 1:1000 in the blocking buffer. The secondary control antibodies, beta actin was diluted in a 1:5000 ratio. [22,23,24] The membrane was removed from the machine and placed in the TBST (1x Tris Buffered Saline, 0.1% Tween 20 Detergent). For the detection phase, a super signal chemiluminescent was applied to the membrane and the membrane was wrapped in a plastic see-through wrap. [25,26,27] The Genesys machine, which was used to determine the result of the western blot detected the result of the primary (Hopx, Ager) and secondary control (b-actin) antibodies. This fluorescence western blotting process provided valuable insights into the protein expression levels of Hopx and Rage in CKO and WT samples, shedding light on potential differences in the pathophysiology of the conditions being studied.

Immunostaining

It is critical to precisely count the amount of AT1 cells in PAH patients and PH mice. [28,29] One such technique is immunofluorescent staining [30], which involves using certain antibodies to identify and count various cell types in lung tissue samples. AT1 cells are thin, squamous epithelial cells that cover most of the alveolar surface and are essential for gas exchange in the lungs. By applying immunostaining, we can use specific antibodies that bind to antigens unique to AT1 cells. This allows for the precise visualization and localization of these cells within lung tissue samples. [31,32] The cell vs tissue protocol was used for both mice and human samples implementing **anti-** HOPX (AT1 cell marker, mouse monoclonal) antibody was used as a primary antibody to locate the AT1 cells within an alveolar space in CKO and IPAH lung tissue. [33,34,35] AT2 cell marker was detected by a primary antibody SP-C (H-8 SC- 51809, lot # D2622 mouse monoclonal) to located AT2 cells within the Alveolar space in an CKO and IPAH lung tissue. Secondary antibodies implemented 594 goat anti-mouse (lgG, lot # 2129448) was used. [36,37,38]

RNASCOPE assay

RNA scope has become an innovative technique that allows for the in person viewing and measurement of RNA at the single molecular level. A new era of molecular diagnostics has begun with RNA scope, which, in contrast to conventional RNA detection methods, offers an unmatched level of sensitivity and specificity. [39,40,41]

RNA scope targets and visualizes specific mRNA transcripts within cells by using a special probe design method and a hybridization-based signal amplification system. With the use of this modern method, we can identify the existence of RNA molecules as well as pinpoint their exact location within a tissue sample. We now have a better understanding of the pathological process by gaining insight into the transcriptional activity of genes. The most recent study in this case used RNA scope's strength to investigate the mystery of IPAH utilizing the Vascular Endothelial Growth Factor A (VEGFA) probe in human samples. IPAH is a subtype of PH, a disease that can cause right heart failure and early death and is characterized by elevated blood pressure in the arteries of the lungs. IPAH and control slides can help us identify whether the VEGFA probe has more activity in either slide. [42,43,44] The initial phase involved preparing the slides. 5 patients diagnosed with IPAH and 5 healthy controls. Target retrieval was used on the slides, which involved submerging them in a citrate buffer solution that was boiling. By dissolving the cross-links created during fixation and releasing the tightly coiled DNA structure, the heat-induced retrieval procedure revealed the RNA molecules within the tissue. [45,46,]

Statistical Analysis

In the study, for the statistical evaluation of the findings, a series of student T-TESTs by the EXCEL software were conducted. Specifically, a T-TEST comparing HOPX and SPC expressions in human controls to IPAH samples, was executed, along with a comparison between mice WT and CKO. Another T-TEST was employed to evaluate the differences in VEGFA expression between human controls and IPAH. A third T-TEST was administered in the findings of quantitative PCR mean for CT values. A statistically significant difference was marked with a P-value of less than 0.05. *(single asterisk): $p < 0.05$, **(two asterisks): $p < 0.01$, ***(three asterisks): $p < 0.001$, ****(four asterisks): $p < 0.0001$ All bar and plot graphs below represent mean±SD.

PAH or Failed Donor	Sex	Race	Age
IPAH1	Female	White	14
IPAH2	Female	White	11
IPAH3	Female	White	39
IPAH4	Female	White	16
IPAH5	Male	White	53
IPAH6	Male	White	13
IPAH7	Male	Asian	29
IPAH8	Male	Asian	18
IPAH9	Female	White	55
IPAH10	Female	White	57
Failed donor 1	Female	White	34
Failed donor 2	Female	White	55
Failed donor 3	Female	White	49
Failed donor 4	Female	White	57
Failed donor 5	Male	White	30
Failed donor 6	Female	White	46
Failed donor 7	Male	Unknown	20
Failed donor 8	Male	White	30
Failed donor 9	Female	White	50
Failed donor 10	Male	White	51

Table 1. Clinical and demographic characteristics of PAH patients and control.

Primer name	Primer sequence
Hopx Forward	AGACTTCCACACGCGCA
Hopx Reverse	AGGATCTCCACCTGGTCCTC
Ager Forward	CACGAGGATGAGGGCACCTA
Ager Reverse	CTCATCGCCGGTTTCTGTGA
Rtkn2 Forward	GGAGCACGGTTAGTGCTGGA

Rtkn2 Reverse	CGAAGCGCTGTGTGCATTCTTT
Aqp5 Forward	GCCATCTTGTGGGGATCTAC
Aqp5 Reverse	AGTAGAGGATTGCAGCCAGG
Cyclophilin Forward	GGCAAATGCTGGACCAAACAC
Cyclophilin Reverse	TTCCTGGACCCAAAACGCTC

Table 2. The primer sequence for qRT-PCR analysis.

Results

Previous Generated Results by Another Lab

In line with the high death rate observed in IPAH patients, $Egln1^{Tie2Cre}$ CKO mice showed a growing trend in mortality, with 80% of them passing away by age of 6 months. It's probable that these mice die to right heart failure, given they display significant RV enlargement, reduced RV contraction ability, and the re-emergence of genes typically seen during embryonic stages in the RV, a pattern commonly found in human heart failure. Interestingly, these mice also exhibit alterations in many genes associated with PH development.[47] RNA sequencing indicates the dysregulation of numerous genes implicated in the pathogenesis of PH, for example, Edn1 (encoding Endothelin-1) and Prkg1 (encoding protein kinase G) in the lungs of these mice Among the 21 genes confirmed through RT-qPCR analysis, 18 are dysregulated in the lungs of Egln1Tie2 mice.[47] Collectively, these findings represent the first mouse model that mirrors the development of clinical PAH.

Figure 1

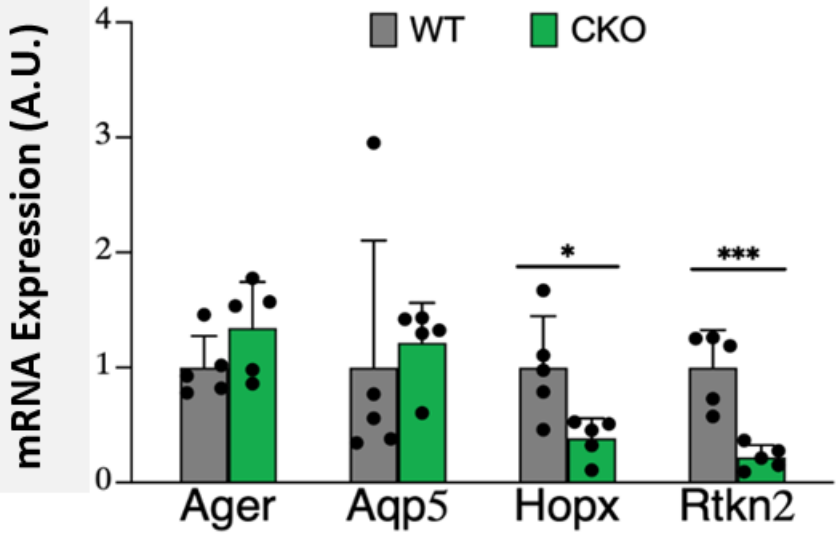

Figure 1. qPCR analysis demonstrated the loss of AT1 cell marker genes.
Whole lung tissue was isolated for RNA and reverse-transcribed into cDNA. qPCR analysis was performed to evaluate the gene expression of AT1 marker genes including Ager, Aqp5, Hopx and Rtkn2 between WT and CKO mice to test mRNA levels. Different expression levels were identified in Hopx and Rtkn2 as the CKO levels show a noticeable change in comparison to the WT. Ager (Rage) and Aqp5 administered an upregulation in the CKO as opposed to the WT. Particularly Hopx and Rtkn2 displayed a downregulation between the WT and CKO, that have the potential for additional study and research. Bar graph with asterisk, AVERAGE (mean), student T-TEST. WT values were normalized as 1. Y-axis mRNA levels are measured in arbitrary units. Data is expressed as mean ± SD. Ager (Rage) p-value > 0.05, Aqp5 p-value > 0.05, *HOPX p-value < 0.05, ***Rtkn2 p-value < 0.001.

Quantitative RT-PCR Analysis revealed a Downregulation of Expression in CKO for HOPX and RTKN2 AT1 Cell Makers in PH Mice Lungs.

To determine if there is a deficiency of AT1 cell expression in PH, we performed qRT-PCR analysis of AT1 cell markers from WT and CKO mice. A detailed measurement of AT1 marker genes can help us better understand if there is a AT1 cells deficiency in PH. By looking at how these AT1 markers are expressed differently, we can gain a better understanding of the cell processes and potential reasons behind PH and the lack of AT1 cells.

We first isolated total RNA from the whole lungs of both male and female WT and CKO mice. RNA was reverse transcribed into cDNA. A list of mice AT1 marker genes including Ager, Aqp5, Hopx and Rtkn2 were selected for qPCR analysis to evaluate the change of AT1 cells in PH. The primers specifically detecting AT1 cDNA were designed using NCBI Primer-BLAST, (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers designed met the following criteria: product size between 70 to 150bp, primers GC content between 40 to 60%, spanning introns, exon junction span if possible.

The qPCR data showed varying and robust expressions of all the AT1 cell makers in all the samples tested. Melt curve analysis showed that there is only one PCR product, suggesting the specificity of the qPCR analysis for the AT1 marker gene. Our data showed that the expression of Hopx and Rtkn2 was reduced in the CKO lungs compared to WT lungs. There was a ~60% reduction in Hopx gene expression and a ~80% reduction in Rtkn2 gene expression in CKO lungs compared to WT lungs. The p-value indicated that there was a significant change in the expression of the AT1 cell markers Hopx and Rtkn2. However, we did not observe significant change of the expression of Ager and Aqp5 between WT and CKO mice. This indicates significant gene expression differences between WT and CKO, which could influence cell reactions in PH, especially considering the precision of qPCR. Previous results mention where both early and late suppressed genes encompassed the majority of AT1-specific genes.

Figure 2

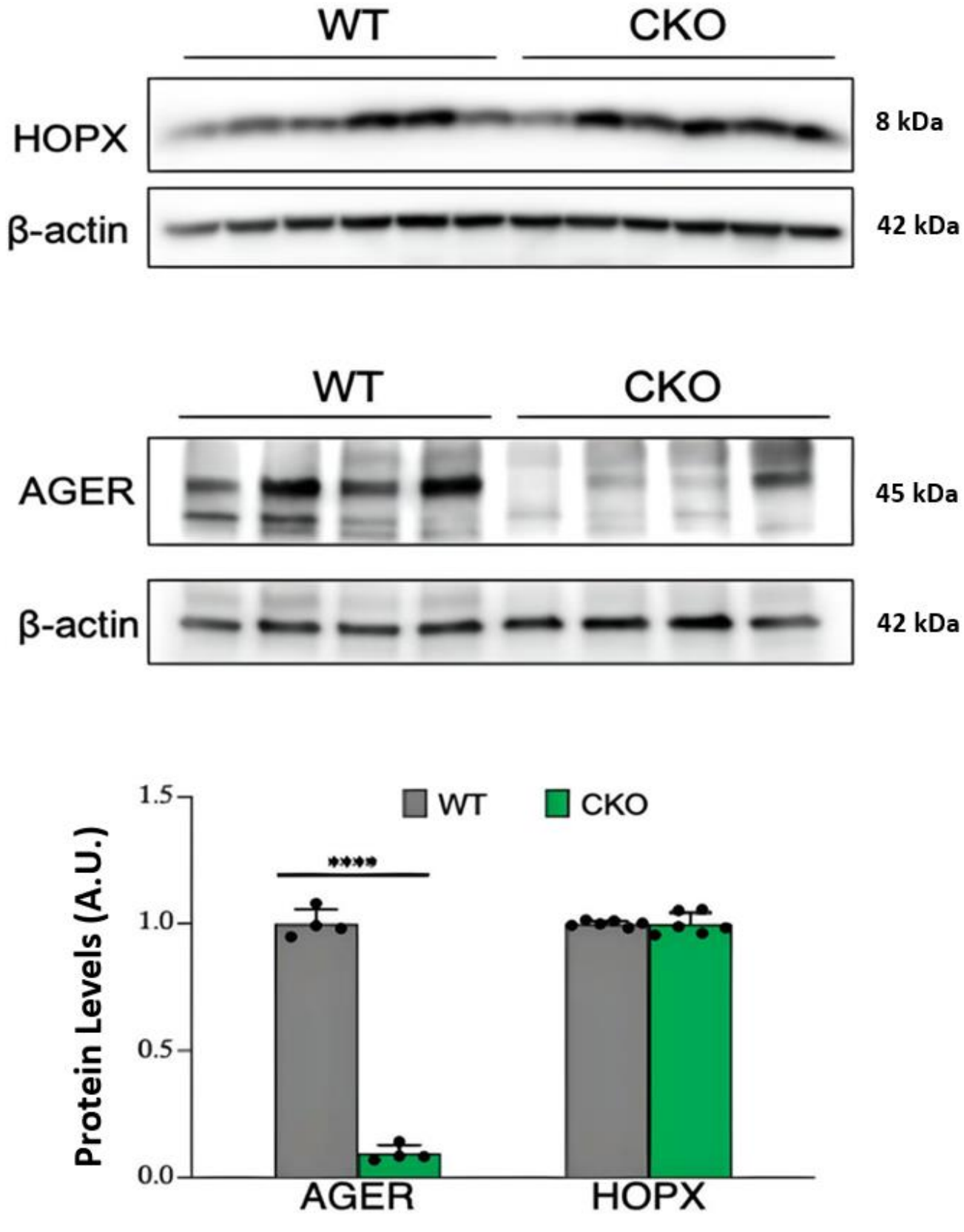
WT
CKO
HOPX
8 kDa
β-actin
42 kDa
WT
CKO
AGER
45 kDa
β-actin
42 kDa
WT
CKO
Protein Levels (A.U.)
1.5
1.0
0.5
0.0
****
AGER
HOPX

Figure 2. AT1 Cell Marker Ager Expression is Downregulated in CKO Mice.

This experiment was designed to fit 6 CKO and 6 WT samples, together with a molecular weight marker. This membrane, post-transfer, was placed in an automated western blot device and treated with primary antibodies for Hopx and Ager at a 1:1000 dilution in the blocking buffer. The internal control antibodies beta-actin, were diluted at a ratio of 1:5000. According to the results, AT1 cell marker Hopx did not show consistency in expression in the WT and CKO mouse samples, beta-actin levels were consistent between CKO and WT mice. Hopx plot indicates little to no difference in AT1 expression. AT1 cell marker Ager did show a noticeable downregulation in expression in the WT and CKO mice samples, beta-actin levels also consistent with CKO and WT. Statistical tools and methods: ImageJ photography edit and marking software, Excel, bar graph, mean±SD, student T-TEST, AVERAGE. Hopx p-value: not significant, ****Ager (Rage) p-value <0.0001 = change detected

Western Blot Analysis Indicated That AT1 Cell Marker Advanced Glycation End Products Receptor (Ager) was Downregulated in CKO Mice.

To analyze the visual expression in blots of AT1 cell markers Hopx and Ager, western blot was performed. Western blotting techniques can help us better understand AT1 expression in the WT control vs the CKO mice. Through this technique, we can gain more clarity on how AT1 cell deficiency is presented in the proteins. The experimental setup was designed to fit 6 CKO and 6 WT samples, in addition to a molecular weight marker. After running the SDS-PAGE gel, the gel was transferred onto a membrane. This membrane, once transferred, was subjected to automatic western blot processing, where it was incubated with primary and secondary antibodies. Following this, the membrane was immersed in TBST (1x Tris Buffered Saline with 0.1% Tween 20 Detergent). For the visualization step, a super signal chemiluminescent agent was applied, and the membrane was then covered in transparent plastic wrap. The results from the western blot, including AT1 markers Hopx and Ager , and internal control beta-actin antibodies, were photographed using the Genesys gel doc machine.

The results of the Western blot analysis using the AT1 cell marker homeodomain protein antibody Hopx indicated that there is no difference in the Hopx expression between the WT and the CKO protein samples. The Western blot was carried out twice particularly for Hopx to validate the outcome. Each repeated experiment yielded consistent results, supporting the preliminary finding that Hopx did not show a difference in the tested samples. AT1 cell marker Ager was successfully detected and showed a difference in the WT and a ~90% decrease in CKO samples utilizing the same method. The control (beta-actin) was consistent through the study for both Hopx and Ager. This suggests that there was no methodological issue that prevented the initial identification of Hopx. The two AT1 antibodies may be regulated differently at the transcriptional, translational, or post-translational level. This suggests that the genetic or environmental changes influencing CKO samples could affect Ager more than Hopx.

Figure 3

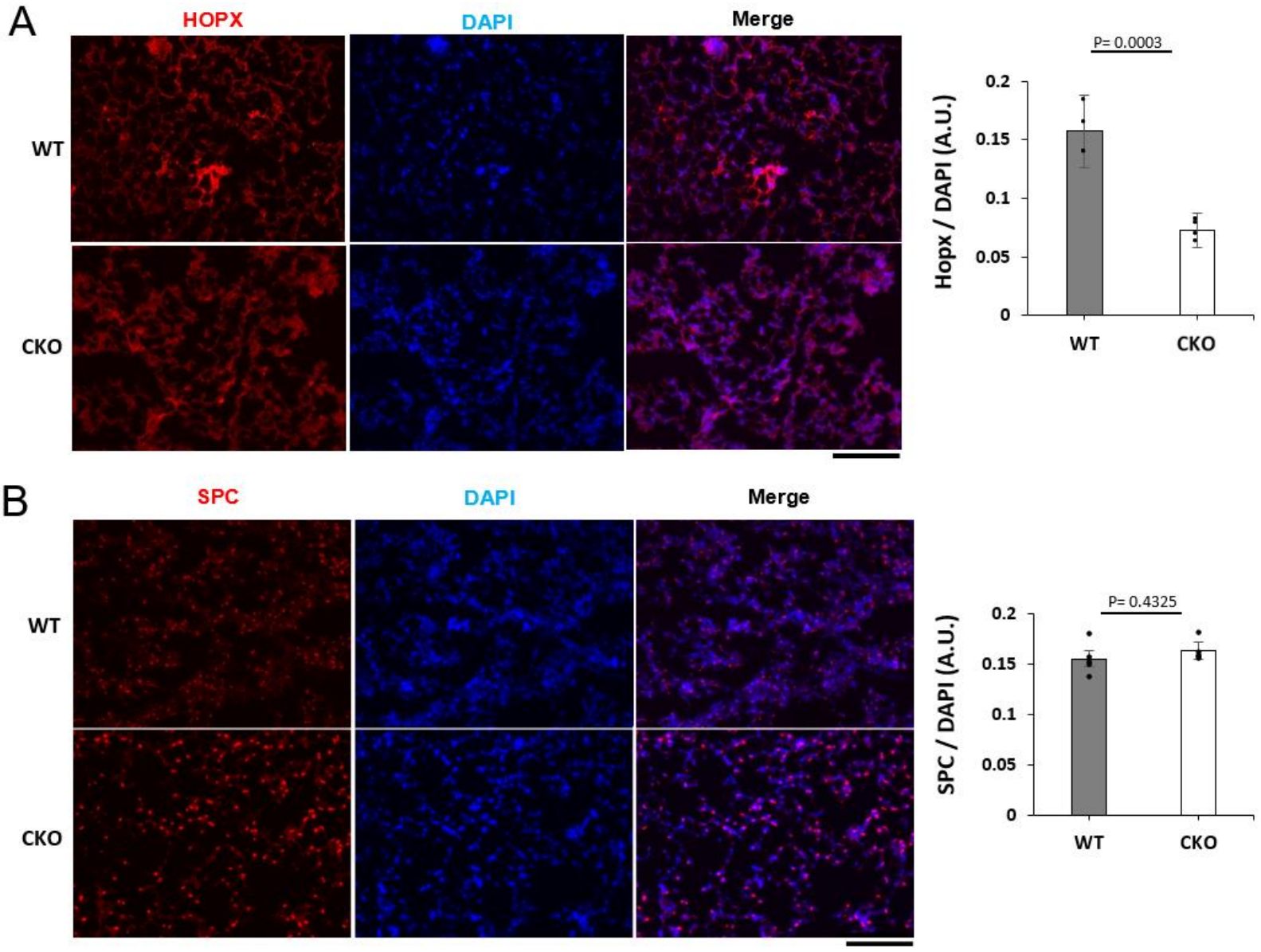

Figure 3. CKO Mice AT1 Cell Count Downregulated in Hopx

A. Immunostaining of Hopx Expression in Mice Lung Tissues 10 mice lung samples (5 WT and 5 CKO) were used in the study. 1:5 antibody dilution factor for each slide and lung tissue. Figure 3 focuses on one Hopx and SPC mouse slide. There is a visual notice in the downregulation of AT1 cell expression in the CKO mice samples as opposed to the WT for Hopx. The CKO mice has a decreased overall expression compared to WT. Scale bar: 130 µm. Mice samples **Hopx Overall p-value<0.001.

B. Immunostaining of SPC Expression in Mice Lung Tissues. After admission of SPC stained AT2 cell samples, WT and CKO values did not show a major change in expression and count between the type 2 cells. Hence rendering the variation between these samples to be insignificant after a student T-Test. Although when comparing SPC to the Hopx, a difference is shown as the Hopx values do show a significant change whereas the SPC values do not show any changes throughout the count. Scale bar: 130 µm. Mice Samples SPC p-value: not significant.

Figure 3A-3B statistical tools and methods: ImageJ photography edit and marking software, ECHO revolve microscope, Excel, bar graph, mean±SD, student T-TEST.

Immunofluorescent Staining Showed a Downregulation of Hopx Stained AT1 Cells in CKO Mice.

To research the deficiency of AT1 cells in-depth, it's crucial to precisely identify and count AT1 cells in a lung tissue sample to record their spread, quantity, and possible changes during the disease. AT1 cells, known for their extensive thin cytoplasm facilitating gas exchange in the lungs, can be specifically identified through immunostaining, a process where antibodies bind to antigens in the tissue, typically marked with fluorescent dyes for visibility under a microscope. Using immunofluorescent staining to view and count AT1 cells within their natural tissue setting allows for a deeper insight into their function, potential downregulations in expression, and involvement in the onset of PH. [48] This experiment wielded 5 CKO and 5 WT mice lung tissue slides. Each slide had 2 different tissue samples, one for SPC stained AT2 cells and the second tissue for Hopx stained AT1 cells. four percent PFA (paraformaldehyde) was administered over the tissues and was let sit for 20 minutes to fix the tissue. Blocking buffer (normal goat serum, 10% Triton, 1x Phosphate buffered Saline) was placed on the slides for 1 hour at room temperature. 1:5 ratio of the primary antibody Hopx and SPC were applied, 1:300 secondary antibody (594 goat anti-mouse) was applied on the second day.

After completing the slide preparations, ECHO revolve microscope, which is used to detect flourescence stained cells, was used to visually locate both AT1 and AT2 cells. ImageJ, a marking and counting software, was also utilized to precisely mark and count the number of cells in the tissue sample. This quantification was then compared by the control WT and the CKO in both SPC and Hopx and a student T-Test was performed to confirm a statistical difference. Our data verified a downregulation of Hopx stained AT1 cells in CKO mice samples. However, the amount of SPC stained AT2 cells showed little to no consistent change in WT and CKO mice samples throughout. The use of immunoflourescent staining to quantify the decrease in AT1 cells aids in understanding the progression of PH. The visual contrast provided by immunostaining highlights the altered lung tissue architecture in PH, offering insights into the pathological mechanisms underlying this condition.

Figure 4

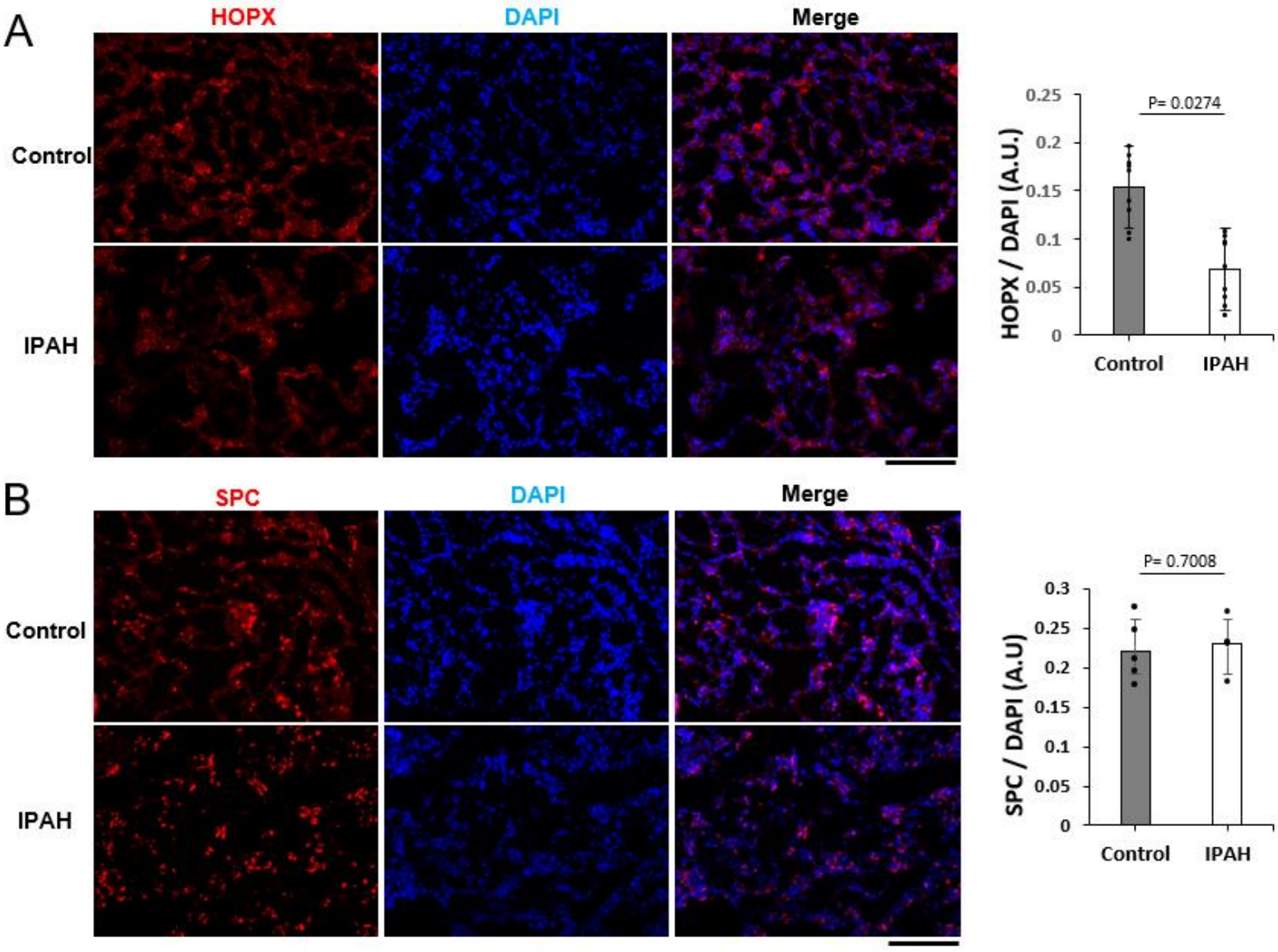

Figure 4: AT1 Cells Were Downregulated in Human IPAH Lung Tissues

A. Immunostaining of HOPX Expression in Human Lung Tissues. A total of 14 slides (7 control and 7 IPAH) were implemented into the study using one section of lung tissue for the AT1 cell marker HOPX. DAPI: blue cell nuclei stain, TXRED: red antibody stain. The first side-by-side section of the lung consisted of HOPX. In the control lung tissue, AT1 cells are abundantly visualized under the staining, suggesting a healthy state with appropriate cellular functionality. However, when IPAH lung tissue is observed under the same conditions, a downregulation in TXRED-stained AT1 cells is evident. This disparity indicates a significant decrease in AT1 cells in the IPAH samples, further affirming the role of these cells in maintaining normal pulmonary function and their potential involvement in the pathogenesis of PH. scale bar: 130 µm. Human samples *HOPX p value<0.05 = change detected.

B. Immunostaining of SPC Expression in Human Lung Tissues. The alveolar space is indicating major positive areas of SPC as shown by the TXRED. The localization of TXRED depends on the specific target

or antibody it is conjugated to. By binding to specific proteins or cellular structures, TXRED can provide insights into the distribution and localization of the targeted molecules within the cell. ST036 -3 IPAH SPC: When comparing the SPC from the IPAH and Control slides, there is not much of a difference when noticing a trend in the SPC count. The SPC antibody shows that AT2 cell count remains the same or similar throughout the lung tissues for both control and IPAH human slides. Scale bar: 130 µm. Human samples SPC p-value: not significant.
Figure 4A-4B statistical tools and methods: ImageJ photography edit and marking software, ECHO revolve microscope, Excel, bar graph, mean±SD, T-TEST.

Immunofluorescence-Staining In Human Lung Tissue Revealed a Noticeable Decrease in AT1 Cells in PAH Patients.

To further confirm the results from the CKO mice staining experiment, we performed additional immunofluorescent staining experiments, utilizing the AT1 cell marker HOPX and AT2 cell marker SPC to evaluate the cellular differences of AT1 and AT2 cells between human control lung tissues and human IPAH lung patients. 14 human slides, 7 control and 7 IPAH lung sections were administered to be tested. The protocol used in the human lung staining experiment was the same as the one used in the figure 3 mice staining. ECHO revolve microscope was used to photograph the images in addition to the imageJ software used to quantify the AT1 cells.

The number of AT1 cells in the human lung tissue samples revealed that AT1 cells were significantly downregulated in IPAH human lung tissues compared to the controls. However, SPC staining results revealed that there was no significant difference in the AT2 cell counts between human IPAH and control samples. The reduction in AT1 cell levels in human IPAH and no significant change in the AT2 cells may indicate that these cells play a significant role in the pathophysiology of PH. [49,50]

Figure 5

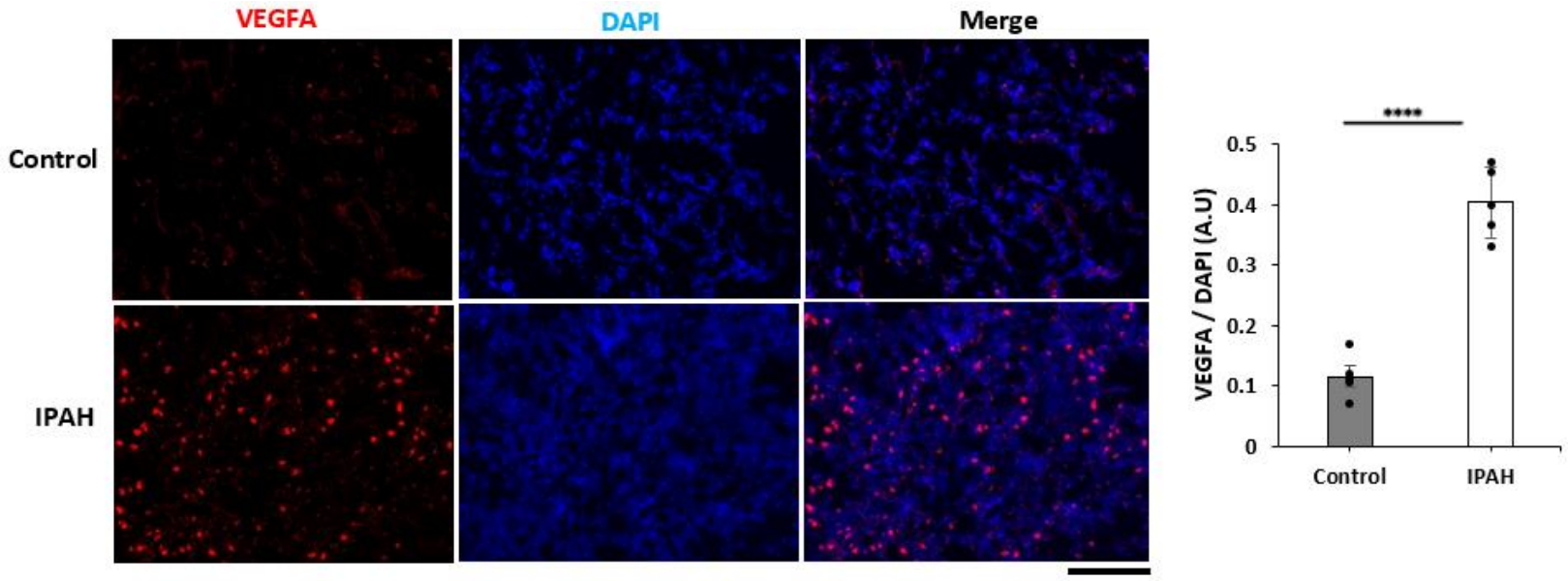

Figure 5. VEGFA Upregulated Expression in Human IPAH lung samples.
Initially, 10 human lung tissue slides were prepared: 5 from patients with IPAH and 5 from healthy control subjects. The slides underwent a target retrieval process, which included placing them in a boiling citrate buffer solution. This heat-based method broke down the cross-links formed during the fixation process and unwound the DNA, making the RNA molecules in the tissue more accessible. More VEGFA expression was observed in the IPAH when compared with the control. DAPI cell nuclei staining showed little to no major difference in IPAH vs the control. VEGFA expression graph shows an upregulation in the IPAH vs the control. Statistical tools and methods: ImageJ photography edit and marking software, ECHO revolve microscope, Excel bar graph, mean±SD, student T-TEST, Scale bar measurement: 130 µm. ****VEGFA p-value < 0.0001

Increased Vascular Endothelial Growth Factor A (VEGFA) expression in IPAH lungs

Since VEGFA is mainly expressed by AT1 cells and considering the decrease of AT1 cells, we hypothesize that due to the downregulation of AT1 cells and cell expression in PH lungs, VEGFA levels will also be downregulated in human IPAH lung samples compared to the control.

Studying VEGFA levels in PH is crucial for several reasons. VEGFA is a key regulator of angiogenesis, the process of new blood vessel formation, which is significantly altered in PH. In addition, it can contribute to the pathological remodeling of blood vessels, as seen in PH. This remodeling can lead to the formation of ineffective, leaky vessels that worsen the symptoms of PH. In this condition, the pulmonary arteries narrow and thicken, leading to increased blood pressure in these vessels and placing a heavy strain on the heart. By examining VEGFA levels, we can gain insights into the underlying mechanisms of VEGFA expressions in PH. The application of RNAscope with a VEGFA probe in studies related to PH is important due to the integral role it plays in vascular biology. [51] By using RNAscope to visualize and quantify VEGFA mRNA expression in human lung tissue samples, we can gain a clearer understanding of the VEGFA expression levels in PAH patients.

The experiment was performed utilizing the FISH (RNA-fluorescence in situ hybridization) protocol. This study compared the levels of VEGFA expression in 5 control human samples and 5 human samples with IPAH using the RNAscope technique and a VEGFA probe. DAPI was applied to count the total number of cell nuclei in each sample. This resulted in a count of 5035 cells in one control sample and 5087 cells in one IPAH sample, demonstrating a similar cell density ratio between the groups. An essential step in the analysis was determining the ratio of VEGFA expressing cells to the total number of DAPI positive cells in each slide. To do this, each slide's number of $VEGFA^+$ cells was divided by the number of DAPI-positive cells on that slide. This ratio gave a measure of the relative fraction of cells expressing VEGFA in each group and allowed for a normalized comparison of the VEGFA expression between the control and IPAH samples. An overall measure of VEGFA expression was created by averaging the ratios calculated for each slide across all the slides in each group. The statistical significance of the observed variations in VEGFA expression was then determined using a student T-TEST on these cumulative values. The p-value was below the significance level of 0.05, indicating that the difference in VEGFA expression between the control and IPAH samples was statistically significant.

Based on the study's results, VEGFA expression was significantly higher in IPAH human samples compared to the controls. The next step would be to investigate the role of how the deficiency of AT1 cells is linked to the upregulation of VEGFA expression in IPAH.

Figure 6

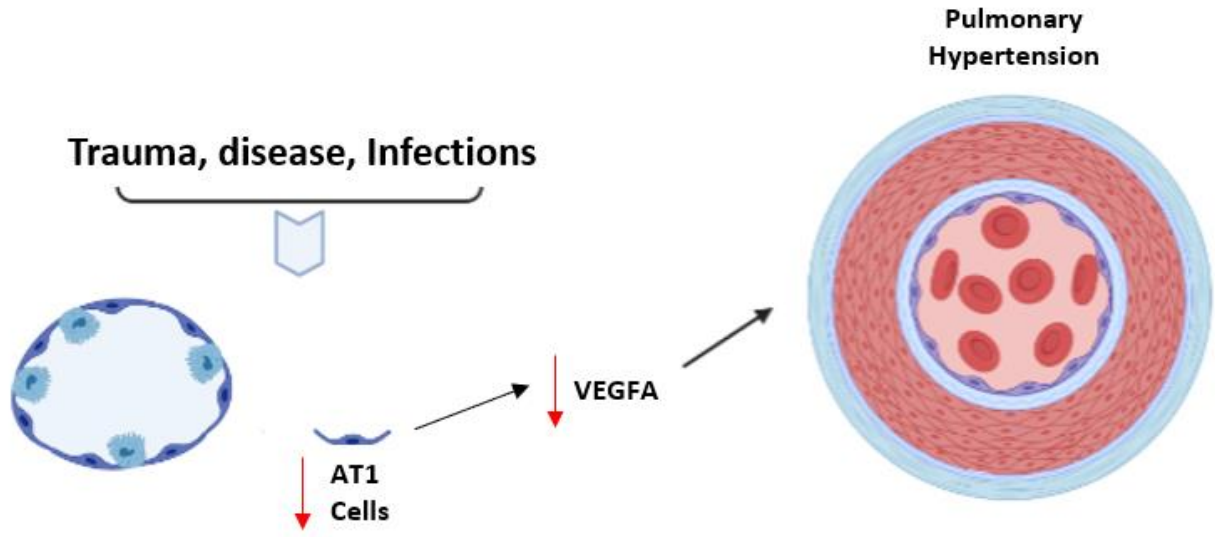

Figure 6. The diagram above visualizes that certain serious trauma, disease, and infections can cause a prominent decrease in AT1 cells that can, in effect, lead to downregulated levels of the angiogenic factor VEGFA, which can then conduct a pathway towards the development of PH. This reduction may play a part in the changes seen in the pulmonary blood vessels due to disease and injuries leading to PH. We also hypothesized that decreased VEGFA expression in PAH patients as opposed to healthy controls raises the possibility that pathological vascular remodeling would be a crucial factor in the illness process and may perhaps be what pushes PH forward.

Discussion

To evaluate the complex dynamics of cellular mechanisms in the context of IPAH. The research carried out in this study used a multidimensional approach. This study offers an innovative model that might serve as an example for future type 1 epithelial cell studies. qRT-PCR analysis revealed a downregulation in the expression of both Hopx and Rtkn2 genes in CKO mice. Conversely, there was a notable upregulation in the expression of Ager and Aqp5 genes. These findings were further investigated using the western blot method where notably, Hopx protein levels did not show a significant change in CKO mice. whereas Ager protein levels were downregulated in the CKO mice, aligning with the qRT-PCR data for Hopx but not for Ager. Furthermore, immunostaining analyses provided additional insights into cellular changes. There was a noticeable decrease in AT1 cells in both CKO mice and human IPAH lung samples. This reduction in AT1 cells is particularly significant, considering their critical role in gas exchange in the lungs. Additionally, in human IPAH samples, VEGFA expression was upregulated. This increase in VEGFA might be indicative of compensatory mechanisms or pathological changes in PH. .[51]

RT-quantitative PCR was used to quantify the expression of AT1 markers, namely Ager, Aqp5, Hopx, and Rtkn2, due to its sensitivity and specificity. The results of these genes helps delineate the behavior and characteristics of AT1 cells in PH. The differential expression of these markers provides a deeper insight into the cellular dynamics and potential mechanisms underlying PH and AT1 cell deficiency. The qPCR results disclosed primers with fluctuating expressions, specifically the AT1 cell markers HOPX and Rtkn2, in the WT and CKO samples. The expression of Hopx and Rtkn2 resulted in change which may be due to a differentiation between Ager and Aqp5, notably associated with proper maintenance of the AT1 cell marker. On the contrary, Ager and Aqp5 results were not downregulated in CKO possibly due to potential cellular stress affecting AT1 cells associated by PH. [52,53,54] These results demonstrate that gene expression levels considerably differ between WT and CKO, may have an impact on cellular responses in PH given the very sensitive and specific nature of qPCR.

While techniques like quantitative PCR provide information on gene expression levels, they don't necessarily convey the protein abundance or post-translational modifications, which can be crucial for cellular function. In the context of AT1 cell markers, understanding the visual protein levels in both WT and CKO mice samples will offer insights into how the knockout affects the synthesis, stability, or degradation of these markers. This is where western blots can reveal the presence of multiple isoforms or modifications of a protein, which might be missed by other techniques (like previously mentioned qPCR). By comparing the protein profiles of WT and CKO samples, we can elucidate the functional consequences of genetic modification, leading to a deeper understanding of the role of AT1 cells and their markers in the pathogenesis of PH. [55,56,57] Ager (Rage is a key player in many pathophysiological processes and is a key modulator of inflammation. It is reasonable to hypothesize that the detected change of the AT1 cell marker may have important consequences for PH's inflammatory responses. This highlights the need for more research into how the interaction between AT1 markers and CKO levels affect PH development. [58] Ager showed a downregulation of expression in CKO samples as opposed to the Hopx, which showed no significance between the CKO and WT.

When investigating PH, it becomes essential to accurately locate and count AT1 cells to understand their distribution, abundance, and potential alterations in the disease state. We will explain the fundamentals of the approach, its benefits, drawbacks, and its possible uses in clinical research and practice as we address the use of human and mice staining to detect AT1 cell changes in samples with PH. By visualizing and counting AT1 cells in their native tissue environment using immunofluorescent staining, a more comprehensive understanding of their role, potential anomalies, and contributions to the pathogenesis of PH can be obtained. In addition, the staining procedure can vary, and the outcomes may be affected by elements like tissue fixation, antigen retrieval, and antibody specificity. To identify AT1 cell loss by staining mice and human samples with the PH phenotype, it has several advantages. It enables high-resolution images that offer precise cell type quantification inside of a tissue sample. It may be carried out using regular laboratory equipment and is rather easy and affordable. It is a common technique for clinical research studies since it can be applied to many different samples and slides. However, there are some restrictions on mice and human staining that must be considered. First, it calls for the utilization of tissue samples, isn't necessarily representative of the entire lung. Although the method utilizes a semi-quantitative approach, it is possible that the number of cells detected do reflect the actual number of cells in the result. Although the method consists of a few drawbacks, it is, however, a crucial tool for evaluating the change of AT1 cells in PAH patients and PH mice. Notably, the findings of the immunofluorescence staining data showed a decrease of AT1 cells in the IPAH human and CKO mice samples, while the SPC stained AT2 cells showed no significant change in expression between the mice WT / CKO and human control / IPAH This data indicates a change in the way cellular systems are affected by PH function. From a clinical standpoint, this would suggest a potential change in cellular connections, which might impair regular physiological functions and affect disease development. [59]

Exploring the expression and localization of critical genes like VEGFA is of paramount importance. VEGFA is a potent mediator of angiogenesis, the process of new blood vessel formation. It promotes the proliferation, migration, and survival of endothelial cells, which line the interior surface of blood vessels. In the context of PAH, the role of VEGFA is complex and multifaceted. Some studies suggest that decreased levels of VEGFA can lead to the abnormal proliferation and constriction of pulmonary arterial cells, contributing to the vascular remodeling seen in IPAH. By utilizing RNAscope for the VEGFA probe, we can obtain a clear spatial representation of VEGFA mRNA expression within lung tissue. This spatial information, combined with the high specificity of the RNASCOPE technique, allows for the identification of the precise cell types and regions producing VEGFA, thereby providing a deeper insight into its role in PH pathogenesis. [60] In our results, VEGFA expression was found to be more active in the IPAH samples by RNAscope analysis. Vascular development and remodeling are crucially regulated by VEGFA, which has consequences for pathological disorders like IPAH. [61] This finding emphasizes the necessity of creating therapy plans that can control VEGFA activity, since they may be significant in controlling PH.

AT1 cell deficiency

The findings of the western blot, Immunofluorescence staining, and quantitative PCR resulted in a difference between the control group and individuals with PH both in humans and CKO mice. The difference showed a deficiency in the expression levels of AT1 cells in the CKO group. AT1 cells help maintain the alveolar epithelial barrier. Deficiency or dysfunction of these cells can lead to increased permeability, allowing fluid and inflammatory cells to enter the alveolar space, leading to edema and inflammation. AT1 cells also contribute to the maintenance of alveolar structural integrity and lung homeostasis and interact with adjacent cells, including AT2 cells, endothelial cells, and fibroblasts. A deficiency in AT1 cells can disrupt this crosstalk, potentially leading to aberrant signaling, inflammation, and fibrosis - processes that can contribute to the pathogenesis of PH. [62]

AT1 and EC cells interaction

AT1 cells and endothelial cells (ECs) in lungs work together closely to promote effective gas exchange, which is a crucial physiological function of the respiratory system. The health of alveolar-capillary barrier and appropriate lung function depend on this interaction. Rapid gas exchange of oxygen and carbon dioxide is made possible by the AT1 cells' thinness, which covers a significant portion of the lung's surface area, and their near proximity to the capillary endothelial cells. Apart from gas exchange, interactions between AT1 and endothelial cells are vital for maintaining the integrity and function of the alveolar-capillary barrier, fluid homeostasis, and alveolar repair. Signaling between these cells, via various paracrine factors and direct cell-to-cell contact, helps regulate processes such as lung development, response to injury, and recovery. Disruptions in the communication or function of either AT1 or endothelial cells can lead to compromised barrier function, resulting in conditions such as PH. The extracellular matrix and basement membrane shared by both cell types, which also permit communication and the exchange of signaling chemicals, help to facilitate this. This connection may be impaired in lung illnesses like PH. AT1 cells are the predominant source of VEGFA, which stimulates the growth of new blood vessels in the alveoli. In addition, VEGFA regulation and Pulmonary blood vessel changes; Influence of pulmonary disease and injury and connection to PAH. Altered VEGFA levels can lead to changes in the pulmonary blood vessels, including remodeling and constriction. A deficiency in AT1 cells can have various implications for lung health and VEGFA as well. Potentially, these changes can ultimately contribute to the development of PH [63]

We employed the RNAscope technique to investigate VEGFA expression in PH compared to controls. Initially hypothesizing that VEGFA expression would be lower in IPAH samples, given its role in angiogenesis and the vascular anomalies associated with the disease. Contrary to the initial hypothesis, the results notably revealed a higher expression of VEGFA in IPAH lung tissues compared to controls. While the results of the experiment contradicted the hypothesis, there is a cause why this specific outcome may have resulted. One such cause is the upregulation of VEGFA caused by other cells. Different cell types (cells apart from AT1) in the lung would compensate for the loss of VEGFA by upregulating it. This response could be an attempt to counteract the reduced angiogenic and capacity in VEGFA associated with AT1 cell loss. Another factor could be endothelial cells substituting the release of VEGFA during a deficiency of AT1 cells. The upregulation of VEGFA in IPAH, despite AT1 cell deficiency, suggests that PH may involve multiple, overlapping pathways that influence VEGFA expression.

Therapeutic potential

The study of a decrease of AT1 cells in PH could hold significant therapeutic potential. AT1 cells are critical for gas exchange in the lungs. Therapies promoting the regeneration of AT1 cells could improve pulmonary function. This could involve strategies for promoting the differentiation of AT2 cells into AT1 cells. For example, stem cell therapy, where regenerating AT1 cells could be experimented with. If AT1 cell loss is found to contribute to inflammation, fibrosis, or vascular remodeling in PH, strategies to prevent or reverse these processes could be explored. The target explored in this research provides mere feedback into how stem cell therapies can be implemented for AT1 cell deficiency. Such as that of immunostaining. Locating where there is a density of AT1 cells in an area of a lung tissue and comparing it to another area where they are not as abundant, can effectively show where a need for stem cell therapy is imminent. Understanding how VEGFA was more present in IPAH samples despite the hypothesis claiming the opposite can also factor in the effect of endothelial stem cell therapy. [64,65]

Limitations

Research into PH is fraught with several challenges that have impeded a comprehensive understanding and effective therapeutic strategies for the disease. Foremost among these is the inherent patient variability in PH, which is stratified by the World Health Organization into five distinct groups, each with its own etiology and clinical presentation. This heterogeneity can significantly impact study results and makes it challenging to develop treatments that are universally effective for each patient. Time restraints further constrain the depth of AT1 cell research experiments, Additionally, variations in counting AT1 and AT2 cells, introduce another layer of complexity and potential inaccuracy to actual results. Addressing these challenges necessitates a multi-faceted approach, including collaboration among researchers and students in research methodologies. Obtaining human samples can also be a limit fraught with several challenges. A smaller sample size can introduce potential biases, reducing the study's power to detect true differences or associations. smaller samples might not capture the full spectrum of clinical presentations or cellular variations associated with AT1 cell deficiency. While initial findings from smaller studies can offer insightful information, they should be regarded cautiously until they are further validated in larger, more diverse groups. The validation of antibodies is another potential limiation. Validating antibodies is a resource intensive process which can be challenging when requiring the antibody for every experiment. In addition, validation results can sometimes be ambiguous or open to interpretation, especially if the antibody produces weak or non-specific signals. Although many limitations for this research can occur, a robust and skillful experimental design is necessary to overlook these limitations and create a new potential for up-to-date insights into PAH pathogenesis and treatment to make the deficiency of AT1 cells in PH a promising area of study in the upcoming future. [66]

Conclusion

This investigation has illuminated a notable deficiency of AT1 cells in both IPAH patients and the PH CKO mouse model. The observed downregulation of key AT1 markers such as Hopx and Rtkn2 in the $Egln1^{tie2cre}$ CKO and the downregulation of HOPX stained AT1

cells in human IPAH and mice CKO underscores the potential importance of AT1 cells in the pathogenesis of PH. The heightened VEGFA activity in IPAH human samples further suggests a significant role for AT1-derived factors in the disease progression. The compromised state of AT1 cells might contribute to a hindered regeneration of the pulmonary vasculature, potentially driving the development and pathogenesis of PH. In shedding light on these relationships, our study opens the door to potential therapeutic approaches focused on repairing AT1 cells and their underlying signaling pathways to treat PH more effectively. Future research into the loss of AT1 cells in PH, and other possible comparable pathological diseases will likely be guided by the abundance of knowledge obtained from this research.

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