It's about time – the role of the biological clock in chronic lung disease, COPD

BY

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Abstract:

Chronic obstructive pulmonary disease is one of the main causes of death nowadays, which is characterized by chronic bronchitis and emphysema, and it is mainly caused by cigarette smoke. In this disease, the epithelium is impaired, the lung stem cell progenitors do not work as they should, and they cannot repair the damaged lung tissue. The circadian rhythm system has been reported to play essential roles in the development of COPD. Circadian rhythms are biological oscillations in mammals with a period of around 24 h driven by the biological clock system. All cells in the human body have their own circadian clocks controlled by a central circadian clock located in the SCN in the hypothalamus. This system has vital roles in the regulation of different body events. In this thesis COPD cell atlas website was used to identify the role of the biological clock in COPD and clarify the core clock genes expression in COPD lungs compared to the control lungs.

The results showed that the expression of these genes was different in COPD lungs than in control lungs. The most interesting result was regarding the CRY1 gene. This gene was increased in the airway cells in COPD while its expression was decreased in AT2A cells. AT2 cells can differentiate into AT1 cells with the help of different signaling pathways, such as WNT/B-catenin signaling. The results also indicated that CRY1 gene was strongly expressed in alveolar cells in control lungs compared to other core clock genes. Therefore, the thesis hypothesized that the CRY1 gene plays a role in the differentiation and proliferation of AT2 cells via WNT/B-catenin signaling. This hypothesis will be studied using the organoids model, and no conclusion can be taken before conducting the experiment.

KEY WORDS: COPD, circadian clock, AT2 cells, CRY1 gene, regeneration.

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COPD: Chronic Obstructive pulmonary Disease.

BMAL1: Brain and Muscle ARNT-Like 1.

PAMPs: Pattern-Associated Molecular Patterns.

DAMPs: Damage-Associated Molecular Patterns.

ARNT1: Aryl hydrocarbon receptor nuclear translocator-like protein 1.

CLOCK: Circadian Locomotor Output Cycles Kaput.

PER1: Period Circadian Regulator 1.

PER2: Period Circadian Regulator 2.

PER3: Period Circadian Regulator 3.

CRY1: Cryptochrome Circadian Regulator 1.

CRY2: Cryptochrome Circadian Regulator 2.

RORa: Retinoic Acid-Related Orphan Receptor alpha

NR1D1: Nuclear Receptor subfamily1, groupD, member1.

EpCAM: Epithelial Cell Adhesion Molecule

AT1: Alveolar Type 1.

AT2: Alveolar Type 2.

SPC: Surfactant Protein C.

TGF-B: Transforming Growth Factor Beta.

ECM: Extracellular Matrix.

IpRGCs: intrinsic photosensitive Retinal Ganglion Cells

SCN: Suprachiasmatic Nucleus.

cDNA: Complementary Deoxyribonucleic Acid.

1. Background:

1.1 Chronic obstructive pulmonary disease COPD:

Chronic obstructive pulmonary disease (COPD) is a severe lung disease associated with older age, and it is generally progressive. COPD is characterized by an inflammation of the airways due to the activation of the innate and adaptive immune system, mucus hypersecretion and airway muscle contraction [1, 2]. This phenotype of COPD is called chronic bronchitis [2]. Besides, emphysema is the most common condition in COPD. It is characterized by a remodelling of the small airway walls, tissue destruction, loss of extracellular elastic matrix and elasticity, and degradation of the alveoli, which lead to a gas exchange problem [2]. These two features of COPD, emphysema and chronic bronchitis, can co-exist in the same patient. The emphysema symptoms are more severe and dangerous and represented by cough, shortness of breath, wheezing, and weight loss [2]. COPD is mainly caused by smoking; a previous study has shown that around 80-90 % of COPD cases are caused by smoking, but other toxic particles and gases also exacerbate COPD [7]. However, a recent study has shown that some COPD patients have never smoked [5]. Nowadays, COPD is a major cause of death; furthermore, no treatment can reverse or even slow down the emphysema that occurred in some COPD patients [9]. Generally, the treatment is based on treating the symptoms and not the disease itself [9]. Although quitting smoking can play a major role in slowing this disease's progression, the damage to the alveoli and airways by COPD is irreversible [2].

This thesis will focus on the epithelial damage in the lower respiratory tract, particularly alveoli by COPD. The epithelium is activated by cigarette smoke or other factors leading to release transforming growth factor TGF-B (excessive), as shown in Figure 1 [1]. TGF-B activates fibroblast proliferation and fibrosis in the small airways, which impairs epithelial repair and leads to airway remodelling [11]. Moreover, alveolar macrophages can also be activated and released pro-inflammatory cytokines, which activates neutrophils [1]. Thus, emphysema is also associated with innate and adaptive inflammatory processes; the innate immune response can be triggered through pattern-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [2]. Both neutrophils and macrophages can release proteases and other enzymes leading to mucus hypersecretion and the breakdown of elastic fibers affecting the elasticity of the alveolar walls [1]. It has also been investigated that a large amount of proteases

disturbances lung protease/anti-protease balance, causing alveolar epithelial cell death by apoptosis [14].

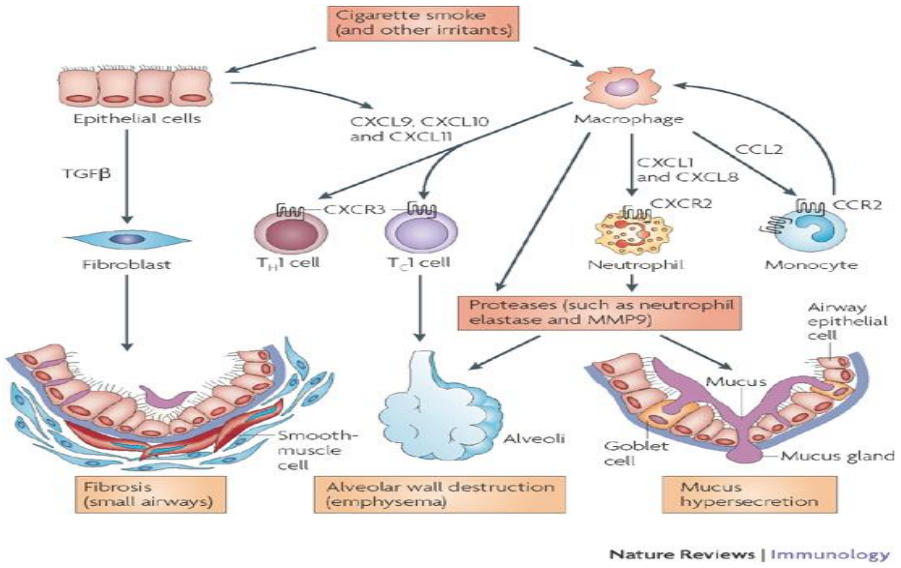

Figure 1. Inflammation in chronic obstructive pulmonary disease (COPD), Barnes et al. (2008). Inhalation of cigarette smoke and other particles leads to the secretion of different chemotactic factors after activation of macrophages and epithelial cells. These factors attract inflammatory cells to the lungs, which ultimately leads to fibrosis, mucus hypersecretion and emphysema [1].

1.1.1 Lung epithelium and Lung regeneration:

There are different cell types found in the airway epithelium, such as ciliated cells, secretory cells and basal cells [3]. Ciliated cells line up the airways, and they are responsible for moving the mucus layer, secreted by goblet cells, and other particles toward the pharynx (upper respiratory tract) [3]. Goblet cells are a type of secretory cells present in large airways, but the secretory cells in small airways are club cells. Club cells produce surfactants such as surfactant protein-A SP-A [17]. Club cells and basal cells function as progenitors for other epithelial cells, including alveolar epithelial cells, and they have an essential role in the alveolar repair process. Therefore basal cells promote the regeneration of the lung epithelial barrier [6]. A recent study has found that club cells in mice act as progenitors upon mild alveolar damage [9].

The alveolar epithelium involves two main types of cells, the first type is AT1 (90% of alveolar epithelium), and the second type is AT2 [8]. The most critical function of this epithelium is the gas exchange [3]. Alveolar type 1 cells make a thin layer with fused basement membranes and capillary endothelium; the gas exchange occurs in this thin layer [3, 6]. AT1 cells are much more prone to apoptosis after injury than AT2 cells; here comes the role of AT2 cells that proliferate and differentiate into AT1 cells, so they act as progenitors for AT1 cells, and AT2 cells also have the ability of self-renewal [15]. Another primary role of AT2 cells is the

production of surfactants that are essential for lung expansion during breathing, such as surfactant protein-C [3, 6]. Like other epithelial cells in the body, lung epithelial cells are connected by an apical junctional complex. Gap junctions facilitate intracellular signals, and tight junctions prevent foreign particles from entering the tissues, this function is also enhanced by the basement membrane [2]. The basement membrane is one of the main compositions of extracellular matrix (ECM), consists mainly of elastin and collagens, and that includes the interstitial matrix as well [12]. Fibroblasts are the main cells that secret this alveolar matrix (ECM) [16].

Lung epithelium cells can ensure the regeneration of the lung with the help of fibroblasts, especially progenitor cells (basal cells, club cells and AT2 cells) that can be activated upon injury and differentiated into desired cell types [7]. Moreover, alveolar epithelial cells are surrounded by mesenchyme cells that promote the differentiation of progenitor cells [2]. It is important to know that the proliferation and differentiation of the epithelial stem cells are regulated by different signaling pathways such as WNT/B-catenin, bone morphogenic protein (BMP), transforming growth factor TGF-β and fibroblast growth factors (FGFs), which play a crucial role in the regeneration of epithelial cells [2]. WNT signalings expressed by fibroblasts can activate the canonical WNT signaling pathways in adjacent AT2 cells, enhancing lung regeneration, as a previous study showed [8]. There are several WNT pathways, and the two most important pathways are canonical (B-catenin dependent) signaling and non-canonical pathway. WNT ligand needs to be linked to the transmembrane receptors of this signalling to ensure the translocation of B-catenin to the nucleus and activate the expression of the B-catenin specific gene, as shown in Figure 2. Otherwise, B-catenin will be phosphorylated by the so-called B-catenin destruction complex and degraded by the proteasome; in this case, target gene expression will be inhibited [43].

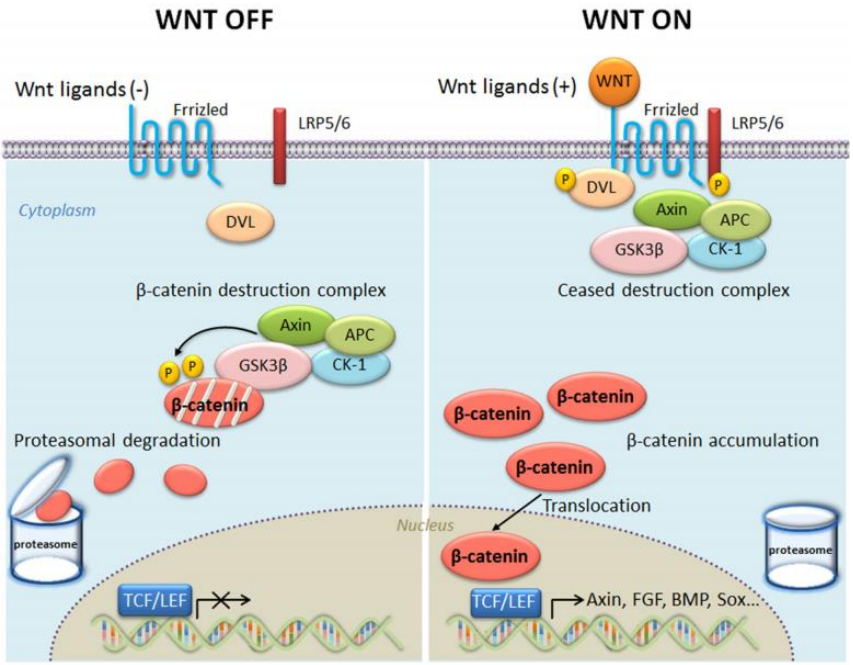

Figure 2. WNT/B-catenin signaling pathway, Ota et al (2016). In the case of activation of WNT signaling, B-catenin can enter the nucleus and activate the WNT gene expression. While without WNT ligand B-catenin will be degraded and the expression of WNT gene will be inhibited [43].

1.1.2 The problem in lung regeneration in COPD:

Epithelial stem cells are vital to repair the damaged lung tissue, and they are essential for lung regeneration after insult, but these stem cells do not work as effectively as they should in the case of COPD due to several changes [12]. The epithelial barrier is considered the first-line defense against inhaled particles, as previously described, but epithelial cell apoptosis has been observed to increase in patients with emphysema, which leads to a dysfunction of this barrier [12]. Moreover, the composition of extracellular matrix ECM, which has a vital role in damaged tissue repair, is affected by COPD [12]. Elastins and collagens could be degraded, and as a result, ECM fragments can enter the blood [12].

Additionally, signaling pathways are abnormally regulated in COPD [16]. A previous study showed that the level of reactive oxygen species (ROS) increased in the lung by cigarette smoking, especially in COPD patients due to the defect in mitochondria [4]. This increase in ROS could affect the signaling pathways needed for stem cell differentiation. The same study showed that the activity of WNT/B-catenin signaling (canonical, B-catenin dependent) could reduce as a result of this incrementation [4]. This pathway is vital in developing AT1 and AT2 cells and reparation of the damage in lung tissue. In contrast, WNT-5A ligand that activates the non-canonical WNT signaling (B-catenin independent) increases in the case of COPD, leading to emphysema development [10]. A study on non-canonical WNT-5A signaling clarified that

canonical and non-canonical WNT signaling pathways are strongly correlated, and WNT-5A could disrupt the WNT-B catenin signaling pathway in COPD [10]. FGF signaling changes have also been reported in COPD; this signaling is needed to develop alveolar regeneration [13]. A mice study showed that FGF-2, which is also called basic FGF, is necessary to prevent emphysema progression in mice that are exposed to cigarette smoke in the short term, but FGF-2 level is reduced in the plasma of COPD patients [13]. It has also shown that the expression of the vascular endothelial growth factor (VEGF) pathway is decreased in COPD patients with emphysema [2]. Another study has shown that TGF-β isoforms are increasingly expressed in the alveolar epithelium in COPD, especially TGF-B1 isoform, which cause lung fibrosis and increasing lung extracellular matrix expression proteins [11].

Recently, the study of the relationship between ageing, cellular senescence, and emphysema development in COPD has a growing interest. Previous study has depicted increased levels of ageing hallmarks in COPD patients, such as higher expression of p21 and p16, telomere shortening, and stem cell exhaustion, which resulted in lung function deterioration and impairing its ability to regenerate [4].

1.2 Biological clock

1.2.1 What is the biological clock, and how does it work?

Humans and other organisms in this life are subjected to environmental changes that are associated with day and night cycles because of the earth's rotation around its axis for 24 hours [20]. These changes are controlled by the biological clock, which generates specific self-sustained oscillations called circadian rhythm [18]. The circadian rhythm helps control different body events, such as cell growth, DNA damage repair, heart rate and body temperature [23]. Early studies proved that all cells in humans have their own circadian clocks, and they are regulated by a central circadian clock found in the suprachiasmatic nucleus (SCN) in the hypothalamus [18, 20]. SCN contains about 20.000 neurons and receives signals from intrinsic photosensitive retinal ganglion cells (ipRGCs) that detect sunlight and other artificial light [18, 27]. SCN transmits these signals to other peripheral clocks via sympathetic and parasympathetic routes (neural pathways) or through releasing hormone, such as melatonin and glucocorticoids, to regulate the sleep-wake cycle, physiological functions, behavior and other functions [19, 20].

Molecular clocks (at a cellular level) consist of specific proteins (transcription factors) and genes. These genes code for proteins that accumulate in the cells overnight and decrease during the daytime [29]. Circadian rhythm is based on transcriptional auto-regulatory feedback loops

regulated by activating elements and repressing elements, as shown in Figure 3 [22, 28]. The activating components are circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1) [18, 22]. CLOCK: BMAL1 complex stimulates the transcription of cryptochrome (CRY1-2), Period (PER1-3) genes and other genes by binding to their Enhancer-boxes (E-box), leading to accumulation of these proteins in the cytoplasm [18]. After that, PER and CRY dimerize and return to the nucleus and block the CLOCK: BMAL1 complex; thus, they block the transcription of PER and CRY genes [18, 22]. CLOCK and BMAL1 are able to promote the transcription of the core clock genes again and start a new cycle when PER and CRY proteins degrade [18]. This mechanism generates 24 h gene expression cycles and provides circadian rhythm at the cellular level. Degradation of these proteins is mediated by the serine/threonine kinases and the F-box proteins [19].

Besides, another transcription loop regulates the transcription of BMAL1, and it includes retinoic acid receptor-related orphan receptor (RORa) that activates the transcription of BMAL1 and REV-ERBa (orphan receptor) that inhibits the transcription of BMAL1. CLOCK: BMAL1 with ROR: REV-ERB also regulates the transcription of D-box binding protein (DBP) [19]. These feedback loops mediate the transcription of clock output genes [19].

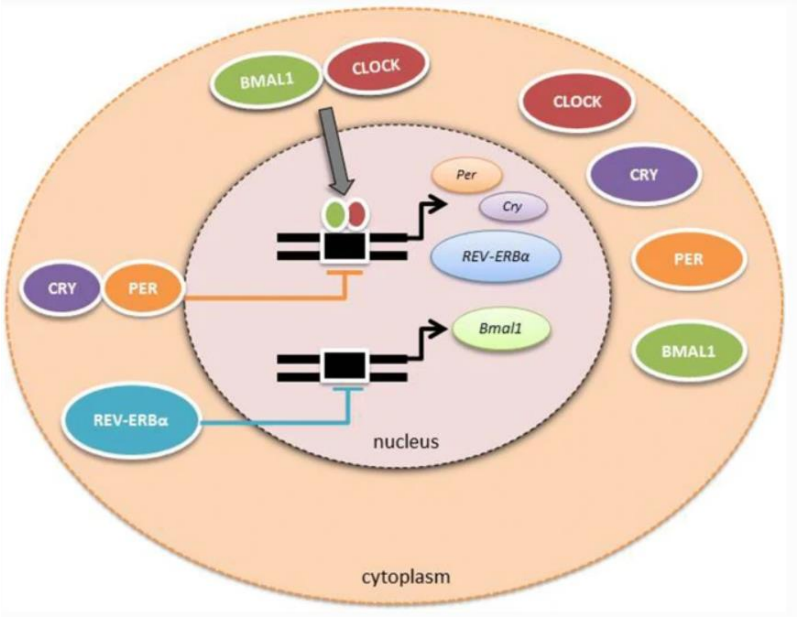

Figure 3. Components of the mammalian circadian clock and transcriptional auto-regulatory feedback loops, Krakowiak et al. (2018). CLOCK: BMAL1 complex activates the transcription of PER1/2 and CRY1/2 genes to give proteins, and when the levels of these proteins increase they will enter the nucleus, repress CLOCK/BMAL1 and inhibit the transcription of PER and CRY [22]**.**

1.2.2 The role of the circadian clock in adult stem cells:

Adult stem cells are able to differentiate and proliferate to give a specific cell type, and they have a functional biological clock [18]. It was shown that when culturing mesenchymal

stem cells in the laboratory, 24-h fluctuations in core clock genes' expression were found [45]. This finding confirmed that the circadian clock contributes to the ability of these cells to regenerate [45]. Furthermore, the adult stem cells homeostasis is regulated by the circadian rhythm, as indicated in a previous study [18]. The regeneration of stem cells is mediated by many pathways, as described before; several studies have investigated that some of these pathways are controlled by circadian clock genes, such as TGF-B and WNT pathways [25, 26]. These studies have also indicated that the circadian clock controls the cell cycle regulatory genes' expression and regulates the cell cycle, which is very important for cellular health [25]. During proliferation, the stem cell cycle comprises four phases: two gap phases G1 and G2, the DNA synthesis phase (S phase), and mitosis (M phase) [25]. After the M phase, stem cells can exit the cycle and start a dormant phase called the G0 phase. At this phase, differentiation of stem cells can occur due to different signaling pathways that can be regulated by the circadian clock, as mentioned before [25].

Given the importance of the biological clock and its crucial functions, it is logical that any disturbance in circadian rhythm could lead to various disorders and diseases.

1.2.3 Dysregulation of the circadian clock in COPD:

The circadian rhythm system is associated with daily changes in the respiratory system. It has a crucial role in lung function regulation; therefore, circadian clock disruption is an essential risk factor for chronic disease such as age-related disease COPD [23]. Several studies have indicated an association between the deterioration in lung function and the dysfunction of the circadian rhythm system in COPD and asthma [23, 19]. Moreover, many of these studies support the fact that different environmental agents such as cigarette smoke can affect the clock function in the lung and accelerate the development of lung diseases, including COPD [19]. Patients with COPD suffer from a disturbance in the circadian rhythm, which indicates that exposure to cigarette smoke affects this clock function in the lung, causing lung injuries and activating the lung's inflammatory process [46]. As clarified previously in this thesis, the major risk of COPD is cigarette smoke; therefore, several studies focused on the effect of CS on the molecular clock in the lung cells.

One of the studies investigated that the CS suppresses the expression of the REV-ERBa gene in mice lungs, and another study showed a decrease of REV-ERBa in small airway epithelial cells in COPD patients, which is related to the development of lung disease [30, 22]. It has also been reported that this gene plays a role in regulating inflammatory mediators [46]. Moreover, previous studies showed a change in clock gene expression and dysregulation of

signaling pathways in mice's lungs exposed to E-cigarettes vapor and a reduction in the lung levels of BMAL1 and PER2 in COPD patients [19, 22]. Besides, it has been reported that mice with PER2 mutant and mice with BMAL1-deficient are more prone to oxidative stress and cellular senescence than normal mice [46]. Furthermore, CS affects clock gene expression via the SIRT1-BMAL1 pathway leading to a reduction in the activity of SIRT1 in the lung epithelial cells and BMAL1 acetylation, which causes emphysema, as a previous study showed [46]. SIRT1 is a NAD-dependent deacetylase, deacetylates BMAL1 and PER 2 proteins and regulates circadian rhythm [19].

In contrast, RORa gene expression is increased in these patients, promoting the development of emphysema [21]. Moreover, CS affects the sleep/wake cycle in the SCN, and it leads to clinical depression; therefore, COPD patients also suffer from sleep disorders and lack of sleep, as COPD symptoms get worse at night and early morning, indicating that the circadian rhythm is disrupted in COPD patients [19, 46]. All these changes in circadian rhythms can affect lung stem cells' ability to differentiate and lead to incorrect functioning of stem cells.

2. Results:

This thesis focuses on the alveolar injuries in COPD, and the relationship between the circadian clock genes and defective lung regeneration in this disease. For this purpose, COPD cell atlas website (www.copdcellatlas.com) has been utilized to obtain more information about the transcriptional changes in core clock genes associated with COPD lungs versus control lungs. The focus was on epithelial and stromal alveolar cells. COPD cell atlas is an open-access database that identifies transcriptional profiles of the different cell populations in COPD. It maps all genes in human lungs and gene expression differences between COPD lungs and control (donor's lungs) [42]. The data on this website was obtained after analyzing the single-cell RNA sequencing of parenchymal lung tissues from 17 patients with advanced COPD and 15 donor lungs [42]. The findings related to the following core clock genes: PER1, PER2, PER3, CRY1, CRY2, BMAL1, CLOCK, RORa and REV-ERBa will be presented and analyzed.

2.1 Expression of core clock genes in the epithelial cells:

The results indicate that following core clock genes CRY1, CRY2, CLOCK, BMAL1 and RORa are highly expressed in the epithelial cells from control lungs compared to other genes PER1, PER2, PER3 and REV-ERBa that are less expressed. Figures 4, 5 and 6 suggest that No aberrant basaloid cells are detected in the control lungs, but they are detected in COPD lungs. These aberrant basaloid cells express CRY1, CRY2, CLOCK, RORa and ARNTL genes.

Besides, there are no differences in the expression of PER1 and PER3 genes in epithelial cells between COPD lungs and control lungs except a slight increase in PER1 gene expression in the ciliated cells from COPD lungs as demonstrated in Figures 4A and 4D. Figure 5A shows that the expressions of the CRY2 genes are generally increased in the epithelial cells from COPD lungs, especially in PNEC cells. Regarding the CRY1 gene, it is clear from Figure 6 that this gene expression is decreased in AT2A cells (distal lungs) in COPD, whereas it is increased in the center airway cells, particularly goblet cells and basal cells. However, the expression of the CRY1 gene is high even in the control lungs compared to other core clock genes, mainly in AT2 cells.

Regarding the ARNTL gene, Figure 4C confirms that its expression is high in the basal cells from control lungs, and this expression is decreased in these cells and PNEC cells in the case of COPD. However, other epithelial cells show a rise in this expression in COPD lungs compared to control lungs. The results, Figure 5C, also represent that the expression of NR1D1 or REV-ERBa gene is lower in the control lungs than other core clock genes, and in the case of COPD, the expression of REV-ERBa gene is decreased. It is evident that the expression of RORa gene is increased in the epithelial cells from COPD lungs compared to the control lungs, as shown in Figure 5B. Finally, the CLOCK gene expression in AT1 cells from COPD lungs is decreased compared to the control; this is also the case of basal cells, ciliated cells, and goblet cells. While this expression is increased in AT2 cells (AT2A and AT2B) from COPD lungs, it is also increased in club cells, see Figure 5D.

Collectively, the data show that the expression of NR1D1 or REV-ERBa gene is strongly decreased in COPD while the expression of CRY1 gene is increased in airway cells in COPD.

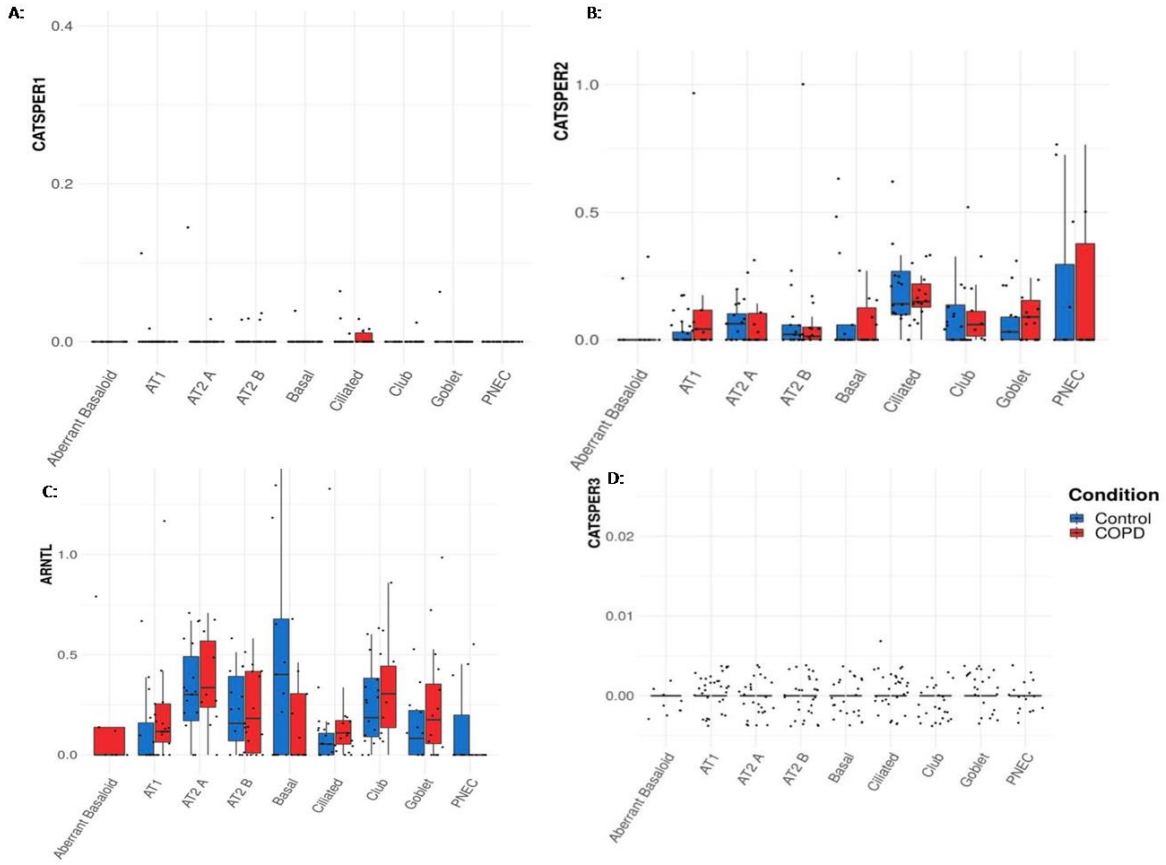

Figure 4. Single cell RNA-SEQ analysis of PER1, PER2, BMAL1 and PER3 expression in the epithelial cells, type of cells is plotted on X-axis against the expression of genes on Y-axis, blue columns stand for control lungs, and red columns stand for COPD lungs. [A] RNA-SEQ analysis of PER1 gene. [B] RNA-SEQ analysis of PER2 gene. [C] RNA-SEQ analysis of BMAL1 genes. [D] RNA-SEQ analysis of PER3 gene.

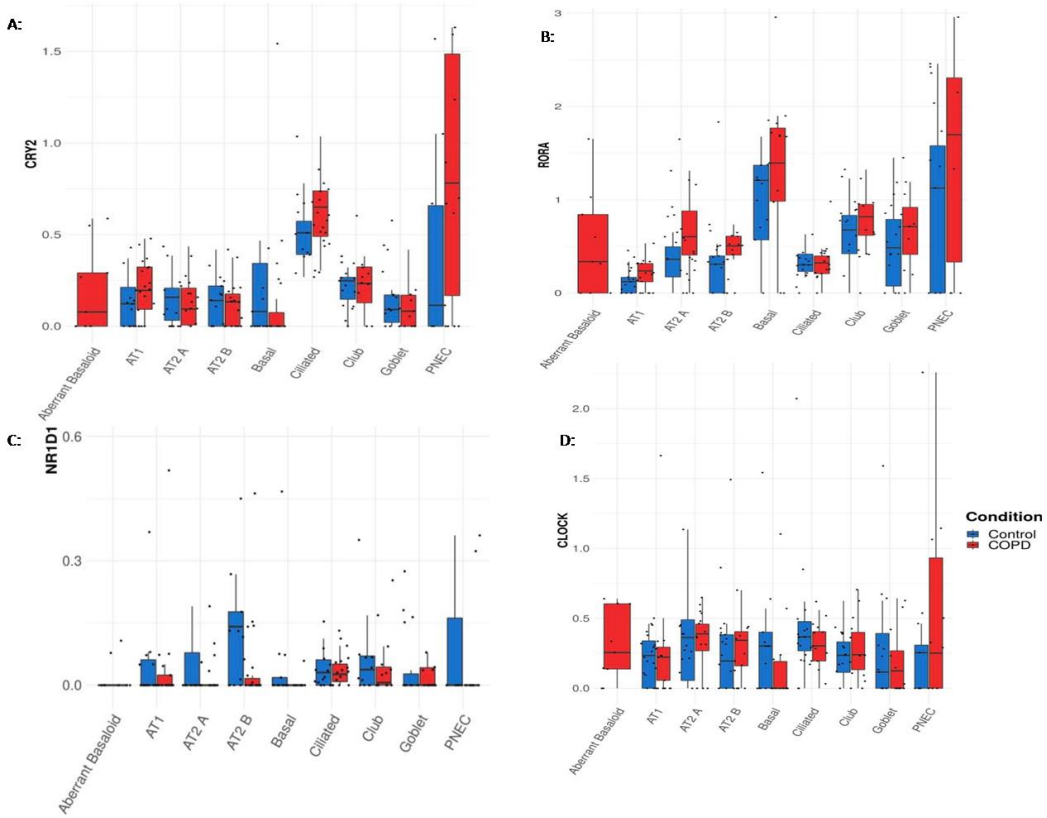

Figure 5. Single-cell RNA-SEQ analysis of CRY2, RORa, NR1D1 and CLOCK expression in the epithelial cells, type of cells is plotted on X-axis against the expression of genes on Y-axis, blue columns stand for control lungs, and red columns stand for COPD lungs. [A] RNA-SEQ analysis of CRY2 gene. [B] RNA-SEQ analysis of RORa gene. [C] RNA-SEQ analysis of NR1D1genes. [D] RNA-SEQ analysis of CLOCK gene.

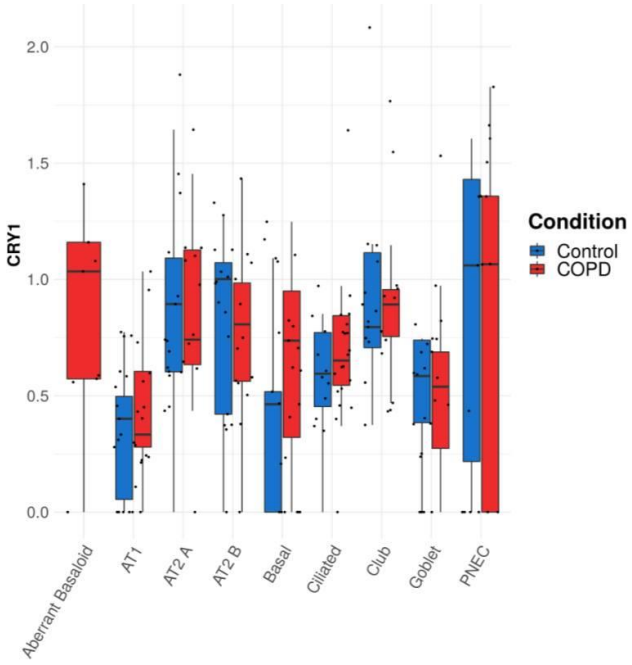

Figure 6. Single-cell RNA-SEQ analysis of CRY1 gene expression in the epithelial cells, type of cells is plotted on X-axis against the expression of genes on Y-axis, blue columns stand for control lungs, and red columns stand for COPD lungs.

2.2 Expression of Core clock genes in the stromal cells:

The most noticeable results are that the core clock gene "RORa" is highly expressed in the stromal cells from control lungs compared to other genes, and the expression of this gene is generally decreased in the case of COPD, as shown in Figure 7. Figure 9D shows that the clock gene is highly expressed in the stromal cells from control lungs except for the fibroblast CTHRC1+ cells that shows less expression of this gene; this is also the case in BMAL1 gene, as Figure 8C illustrates. However, Figure 9D indicates that the clock gene expression is increased in some stromal cells from COPD lungs, including fibroblast CTHRC1+ cells, while it is clearly decreased in the alveolar fibroblast. Also, the results do not show any differences in the expression of PER1 and PER3 genes between the stromal cells from COPD lungs and the same cells from control lungs; see Figures 8A and 8D. Moreover, CRY1 gene expression is increased in most stromal cells in COPD, while the expression of CRY2 genes is primarily increased in the fibroblast CTHRC1+ cells that expressed more CRY2 gene in COPD lungs, as seen in Figures 9A and 9B.

Collectively, the data show that RORa gene and CLOCK gene are higher expressed in the stromal cells from control lungs than other clock genes.

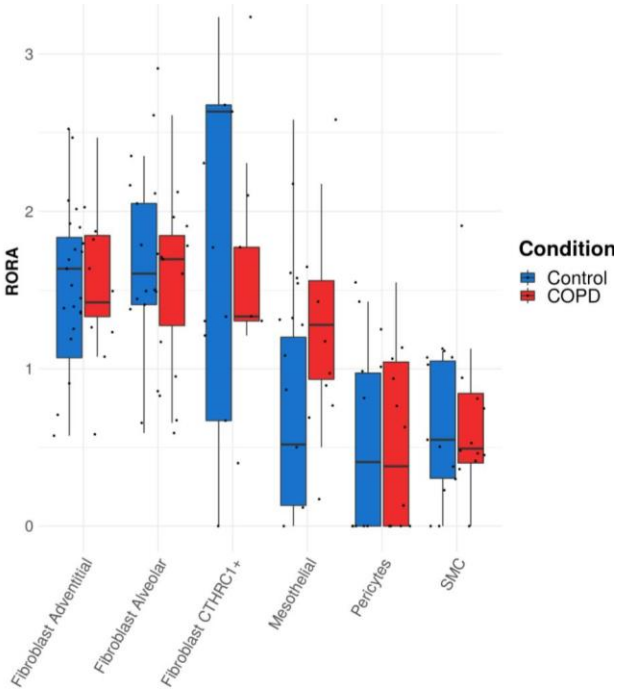

Figure 7. RNA-SEQ analysis of RORa gene expression in the stromal cells, type of cells is plotted on X-axis against the expression of genes on Y-axis, blue columns stand for control lungs and red columns stand for COPD lungs.

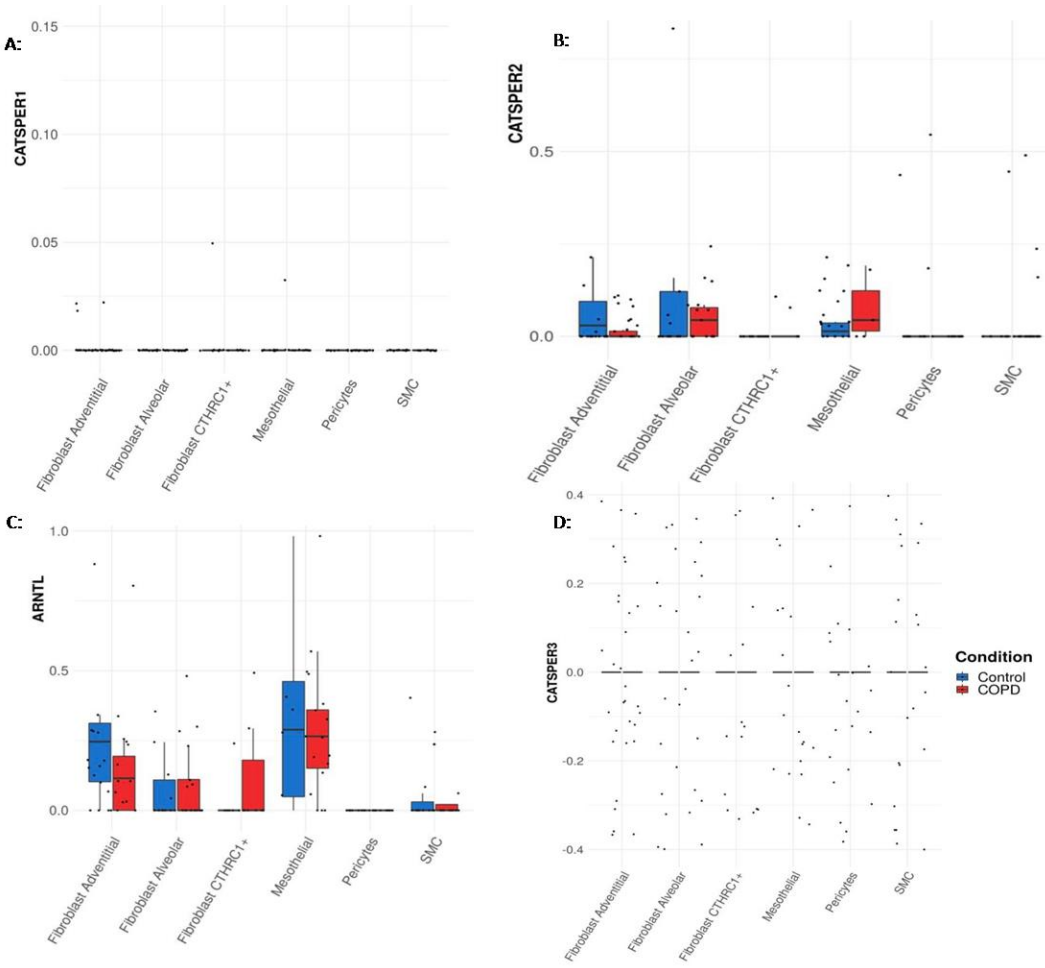

Figure 8. RNA-SEQ analysis of PER1, PER2, ARNTL1 and PER3 expression in the stromal cells, type of cells is plotted on X-axis against the expression of genes on Y-axis, blue columns stand for control lungs and red columns stand for COPD lungs. [A] RNA-SEQ analysis of PER1 gene. [B] RNA-SEQ analysis of PER2 gene. [C] RNA-SEQ analysis of ARNTL gene. [D] RNA-SEQ analysis of PER3 gene

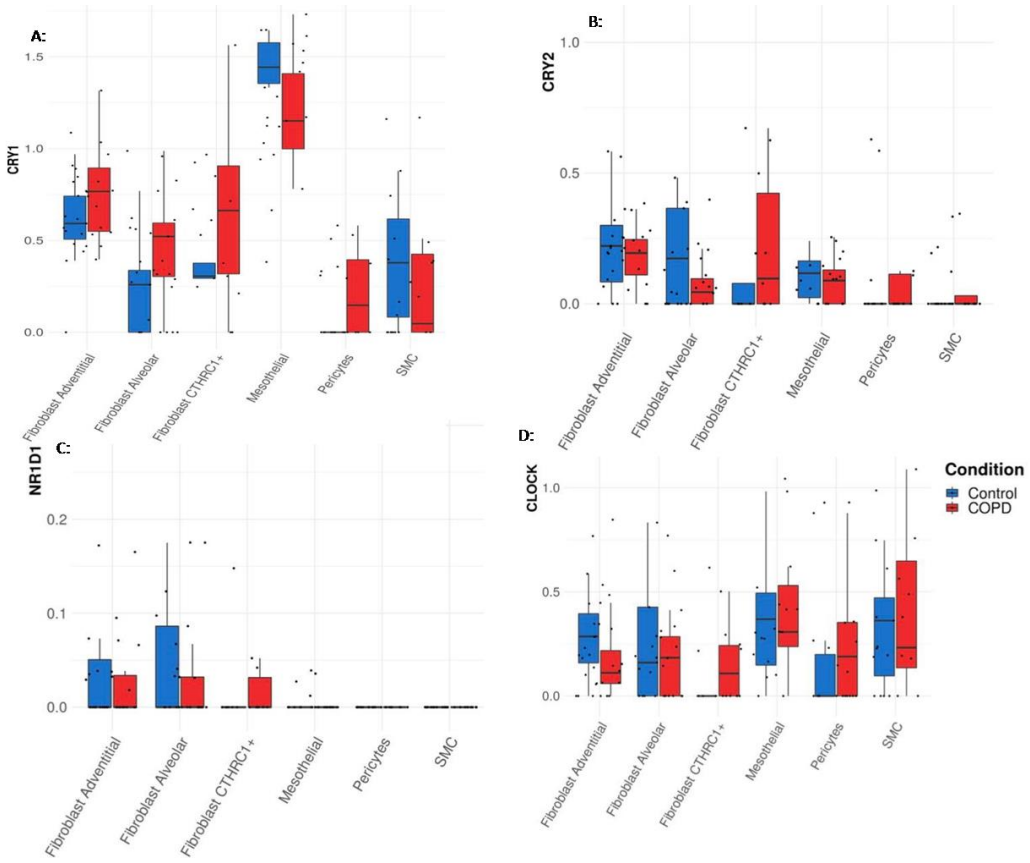

Figure 9. RNA-SEQ analysis of CRY1, CRY2, NR1D1 and CLOCK expression in the stromal cells, type of cells is plotted on X-axis against the expression of genes on Y-axis, blue columns stand for control lungs and red columns stand for COPD lungs. [A] RNA-SEQ analysis of CRY1 gene. [B] RNA-SEQ analysis of CRY2 gene. [C] RNA-SEQ analysis of NR1D1 gene. [D] RNA-SEQ analysis of CLOCK gene.

2.3 Difference between epithelial and stromal cells in the expression of core clock genes:

Figures 10 and 11 show the differences in the core clock gene expression in the epithelial and stromal cells in control lungs and COPD lungs. These Figures indicate that core clock genes CLOCK, CRY1, PER1 and PER2 genes are higher expressed in the epithelial cells than the stromal cells, in contrast to the RORa gene that is higher expressed in the fibroblasts than the epithelial cells. There are no significant differences in the expression of ARNTL and NR1D1 genes between these two types of cells.

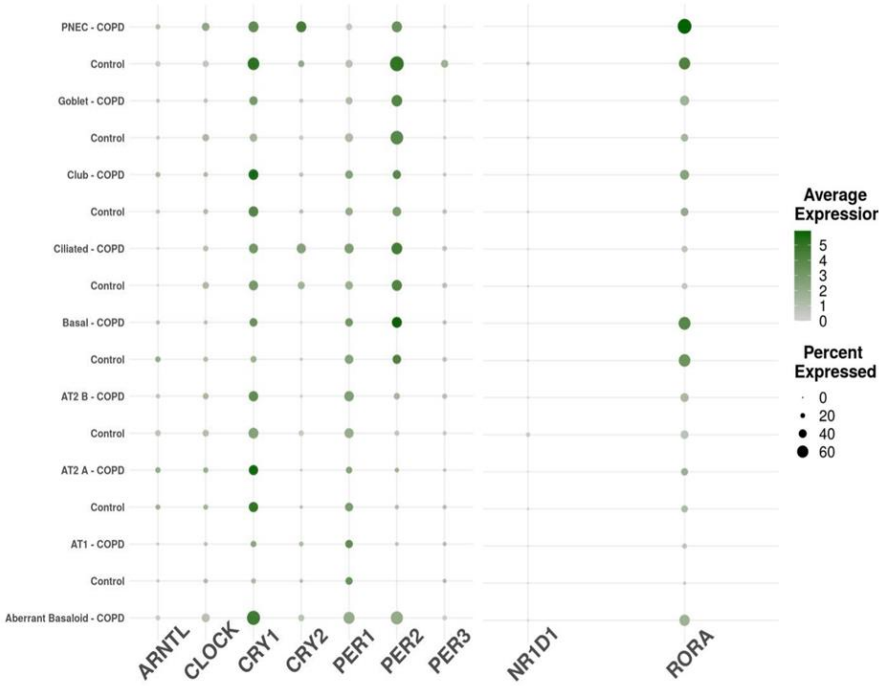

Figure 10. Dot plot of core clock genes expression in the epithelial cells. The plot's size is related to the percentages of gene expression, and the colour reflects the degree of the expression.

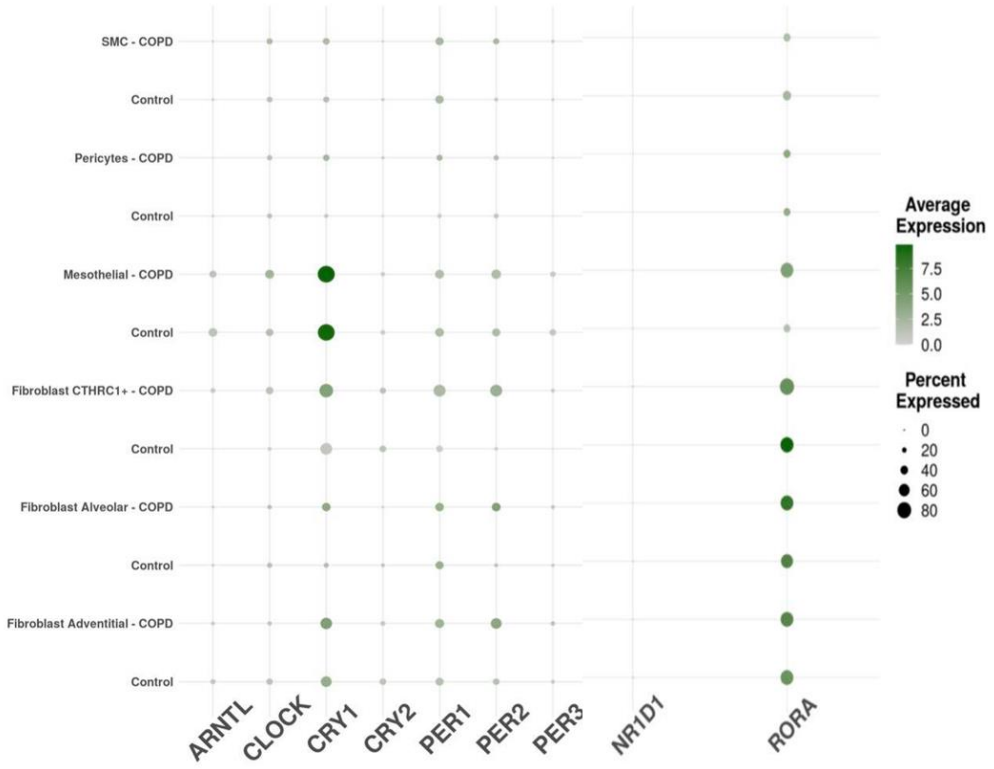

Figure 11. Dot plot of core clock genes expression in the stromal cells. The plot's size is related to the percentages of gene expression, and the colour reflects the degree of the expression.

3. Discussion:

COPD disease is caused by repeated injuries to the epithelium associated with decreased activity of fibroblasts leading to the destruction of alveoli and emphysema [16]. There is growing evidence that disturbances in the circadian rhythms are implicated with the accelerated development of emphysema [23]. In this thesis, we used COPD cell atlas to understand the association between core clock genes expression and COPD in the epithelial and stromal cells. The results indicated differences in the expression of the core circadian genes in COPD lungs compared to the control lungs, in both epithelial cells and stromal cells. That was expected because several studies have reported abnormal circadian rhythm in COPD, as mentioned previously [19, 22]. As shown in the results, Figure 5B, the RORa gene expression was strongly increased in the epithelial cells from COPD lungs. This discovery is consistent with a previous study conducted on COPD lungs, which also reported an increase in the RORa gene in these lungs [21]. Researchers in this study indicated that an increase in RORa gene expression in COPD enhanced cell death and apoptosis of epithelial cells [21]. Another interesting finding is that the REV-ERBa gene was decreased in COPD lungs in both epithelial cells and stromal cells; this has also been proven by previous studies that reported a vital role of the REV-ERBa gene in cell proliferation [22, 30]. These abnormal levels of RORa and REV-ERBa were implicated in inflammation, increased DNA damage and cellular ageing in COPD [24]. Recently, it has been shown that REV-ERBa has a protective role and can prevent the progression of pulmonary fibrosis [49].

The most remarkable result is that the expression of the CRY1 gene was increased in basal cells and goblet cells (airway cells) and decreased in AT2A cells (distal lungs) in COPD. COPD is generally characterized by emphysema, which is associated with the loss of AT1 and AT2 cells (distal lung cells), whereas in airways, COPD is characterized by hyperplasia of basal cells and an increase in the number of goblet cells [2]. Therefore, it can be hypothesized that the CRY1 gene could play a role in the proliferation and differentiation of the epithelial cells. Besides, the increase in CRY1 expression in the airway cells could be associated with increasing these cells in COPD. On the other hand, the decrease in CRY1 gene in AT2 cells could also be related to AT2 cells loss in COPD.

Moreover, the CRY1 gene is strongly expressed in AT2 cells compare to other core clock genes in healthy lungs, and given the function of these cells; it can be hypothesized that the CRY1 gene could promote the differentiation of these cells. CRY1 gene is the most critical component of the biological clock, and it is generally recognized as the major repressor of the transcription of

CLOCK and BMAL1; a change in the expression of this gene may affect the expression of other clock genes and affect the physiological processes of the body as reported in a previous study [32]. It has been shown in a study on intestinal stem cells that CRY1 genes play a role in the reproduction and division of the mammalian stem cells directly by influencing the cell cycle, and lack of this gene suppresses these processes [33]. Besides, CRY1 genes have an essential role in the proliferation of neurospheres, and the loss of this gene is associated with apoptosis [33]. It has also been reported that a decrease in CRY1 expression in hepatocytes, which are the epithelial cells of the liver, has a negative effect on the cell cycle, as CRY1 plays an important role in the proliferation of hepatocytes [47]. Another study confirmed that the expression of the CRY1 gene increases during adipogenic differentiation [34]. The same study reported a vital role of CRY1 genes in the differentiation of these cells via canonical WNT/β-Catenin signaling [34]. The expression of the WNT signalling genes is affected by the dysregulation of the circadian clock, as previously indicated [38]. Moreover, a previous study on osteoblasts found that CRY1 gene overexpression improves the differentiation of these cells in vitro and in Vivo [32]. This study also reported a relationship between CRY1 gene and canonical WNT/β-Catenin signaling, as the increase in CRY1 gene levels is accompanied by an increase in this signaling activity [32].

Notably, several studies have indicated that the CRY1 gene is associated with the development of several epithelial cancers, such as colorectal, breast and prostate cancer, where disturbances in the expression of the CRY1 gene has been reported in most cases. As known, almost all epithelial cells contain stem cells, which are responsible for tissue regeneration after insult. Table 1 reports different studies regarding the role of CRY1 gene in the development of epithelial cancers.

Table 1. The role of CRY1 gene in different epithelial cancers.

Type of cancer	CRY1 expression	The role of CRY1 gene
Colorectal cancer [39]	The expression of the CRY1 gene was increased in most colorectal cancer cells.	CRY1 gene enhanced human colorectal carcinoma cells (HCT116) proliferation.
Prostate cancer [40]	CRY1 gene was increased in prostate cancer, in particular in the late-stage of this disease.	The CRY1 gene enhances cell proliferation and DNA repair processes making cancer cells more resistant to treatment.
Ovarian cancer [41]	In ovarian cancer, the expression of the CRY1 gene was significantly higher than in normal ovaries, while the expression of	The role of the CRY1 gene in this cancer is still unclear.

	other core clock genes was lower in cancer.	

As described in the background section of this thesis, the differentiation and regeneration of epithelial cells, particularly the differentiation of AT2 cells to AT1 cells, are regulated by different signaling pathways. The most critical pathway in this process is the WNT signaling pathway. The primary molecule of this signaling is B-catenin which has an essential role in the proliferation and differentiation of stem cells [1]. COPD is associated with the imbalance between canonical and non-canonical WNT signaling with an increase in WNT-5B that inhibits the differentiation of epithelial cells [35]. As mentioned previously, several studies indicated a relationship between this signaling and CRY1 gene, especially concerning the differentiation of stem cells [32, 34]. Based on the previous results and explanations, the hypothesis of this thesis will be as follow: Circadian gene cryptochrome 1 promotes the proliferation and differentiation of alveolar epithelial cells type 2 via WNT/B-Catenin signaling. Therefore, these thesis objectives are to investigate the role of the CRY1 gene in the differentiation of alveolar epithelial type 2 cells and to identify the relation between the CRY1 gene and the WNT/B-Catenin signaling pathway during lung regeneration in healthy lungs and COPD lungs.

4. Methods:

To examine my hypothesis, I would like to use a combination of in vitro and in vivo experiments with the help of different techniques: 3D organoid culture System, Cre-LoxP technique and Immunofluorescence. Organoids models will be generated from mice exposed to air or cigarette smoke in vivo before (control) and after knockout CRY1 gene specifically from AT2 cells using the Cre-LoxP system to determine the role of the CRY1 gene in the differentiation and proliferation of AT2 cells. The effect of the WNT/B-catenin signaling pathway in the growth of the organoids and its relation with the CRY1 gene will be studied using organoids generated from mice exposed to air or cigarette smoke in vivo when adding WNT/B inhibitors to the organoids medium. The size and the number of the organoids (SPC) and nucleus B catenin levels will be measured using Immunofluorescence, and the level of CRY1 expression will also be measured using IF; the experiment design is presented in Figure 13.

4.1 Principles of techniques:

4.1.1 Organoids model[36]:

An organoid lung is a small lung designed with three-dimensional (3D) tissue that reconstructs the functions of lung tissues in vivo and mimics the in vivo environment. This technique is

derived from stem cells (progenitor) and can be used to follow lung epithelium regeneration in vitro [36]. This model is essential for studying several biological processes, cells interaction and the effect of different diseases in the cells [36]. I would like to use this model in my experiment to follow the regenerative function of AT2 cells and how this function can be affected by different conditions (air exposure, cigarette exposure, CRY1 knockout and inhibition of WNT/B catenin).

4.1.2 Immunofluorescence[31]:

This technique is based on the fact that certain antibodies can bind to the target molecule, and this complex can be couple with fluorescent dyes that make this molecule visible under the microscope. Indirect Immunofluorescence will be performed in my experiment, which is composed of several steps: Fixation, blocking, primary antibody incubation, second antibody incubation and staining [31].

In this experiment, the B-catenin levels in the nucleus will be measured because B-catenin is expressed in the epithelial membrane, and when the WNT pathway is active, it translocates to the nucleus, but the total B-catenin may not necessary to change, as described in theory. Therefore it is better to focus on the nucleus levels of B-catenin [43].

4.1.3 Cre-LoxP strategy [37]:

In this technology, there is an enzyme called Cre-recombinase isolated from bacteriophages P1 and targets specific DNA sequences called LoxP sites. In order to get a transgenic mouse with an activated Cre-LoxP system, two transgenic mice are needed. The first mouse is with Cre protein controlled by a promoter, a tissue-specific promoter, and the second one with the target gene flanked with two LoxP sites facing one way, as shown in Figure 12. The target transgenic mouse will be obtained after crossing these two mice. This mouse has both the Cre enzyme with the specific promoter and the target gene between two LoxP sites. The Cre enzyme will digest the target gene only in the cells that expressed the used promoter.

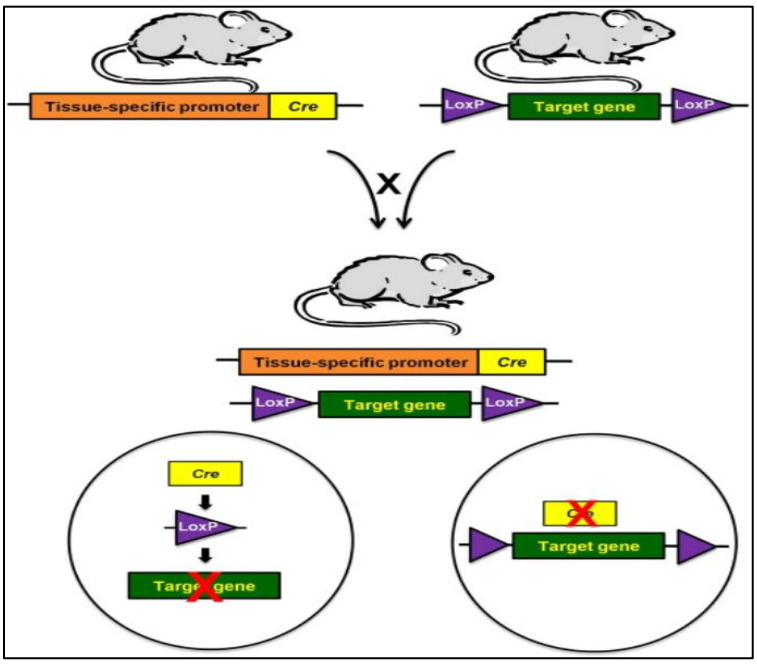

Figure 12. Cre-LoxP system, Harno et al (2013)[37].

4.2 Experiment:

I would like to use a protocol from the published articles of the Department of Molecular Pharmacology at the University of Groningen [35] [44]. First, I would like to remove the lungs from adult mice using magnetic cell separation and then lung single-cell suspension will be prepared by proteolytic digestion of the lungs. Afterwards, I will make a negative selection of CD31 cells and CD45 cells. Finally, I will make a positive selection of EpCAM cells which are lung epithelial cells. These cells will mix with equivalence numbers of fibroblasts (CCL206), one-to-one ratio and seed in Matrigel for fourteen days. After 14 days, I will access the size and the number of the organoids model, and measure the levels of CRY1 gene and nucleus B-catenin using Immunofluorescence (IF). The previous protocol will be repeated several times: organoid from adult mice exposed to air in vivo, organoid from adult mice lungs exposed to air in vivo after knockout CRY1 gene from AT2 cells, organoid from adult mice lungs exposed to air in vivo with adding WNT/B inhibitor to the organoid model. I would like to use a tankyrase as a WNT inhibitor, which works by accelerating AXINE2 knockdown and blocking the function of B-catenin, and I will add it to the organoids medium on day 7 after co-cultured EpCAM+ cells with fibroblasts [50]. After that, the same steps will be repeated, but after exposing the mice to cigarette smoke in vivo for four days.

For immunofluorescence staining, I would like to use a protocol of the same mentioned group [35, 44]. First, I will remove the medium from organoid culture, and then I will fix it for

15 minutes with acetone and methanol [1:1] at −20°C. After that, I will add Phosphate-buffered saline (PBS) with 0.02 % sodium azide, and I will keep them for one week at 4°C. I would like to use bovine serum albumin (BSA) as a blocking agent, and I will add it to the medium for 2 hours. After that, the cells will incubate with primary antibodies and PBS buffer with 1% BSA and 0.3% Triton X-100 (CRY1 antibody, anti-pro-SP-C antibody and b catenin antibody) overnight at 4 °C. The next day the cells will wash three times using PBS and at room temperature they will incubate with secondary antibody (Donkey anti-rabbit IgG Alexa Fluor®488) for 2 hours. I will also wash with PBS three times after 2 hours from incubation with secondary antibodies, and then I will prepare nuclear staining by incubating with 4′, 6-diamidino-2-phenylindole (DAPI) for 15-20 minutes. The samples will be washed using PBS and UP water then they will be ready for imaging using light microscopy at 20x magnification, a Leica SP8 microscope or a Leica DM4000B microscope [35, 44].

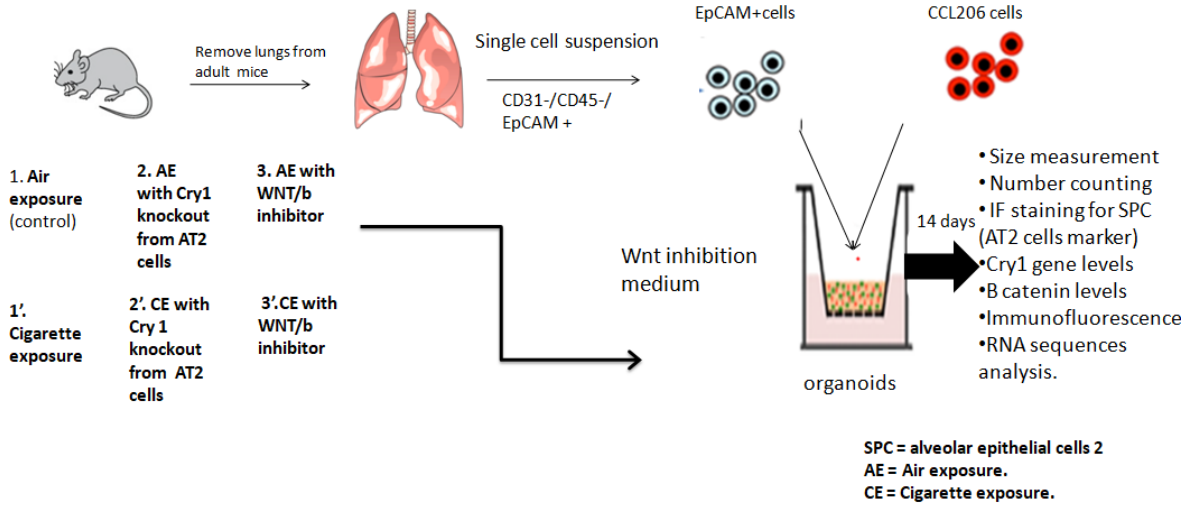

Figure 13. Experimental design.

To generate transgenic mice with deleted expression of CRY1 gene specifically in alveolar type 2 cells, I would like to use the Cre-recombinase technique in which Cre enzyme will be driven by surfactant protein C. Surfactant protein C is highly expressed in AT2 cells, and this ensures that the gene is only deleted in these cells. SPC-Cre transgenic mice will be generated using cloned complementary deoxyribonucleic acid (cDNA) contains a nuclear localization signal downstream of the 3.7-kb human SPC promoter sequence and Cre recombinase [48]. Then I will cross these mice with mice containing the CRY1 gene flanked by two lox P sites to get the required mice.

5. Conclusion:

The results of the COPD cell atlas suggested that the expression of core clock genes was changed in COPD lungs compared to the control lungs. The expression of the CRY1 gene was increased in airway cells in COPD while it was decreased in AT2A cells. This gene expression was also high in the epithelial cells, particularly AT2 cells in the control lungs. AT2 cells differentiate into AT1 cells upon injury and this process is regulated by several signaling pathways, such as WNT/B-catenin signaling. Therefore I hypothesized that CRY1 gene plays a role in the differentiation of AT2 cells via WNT/B-catenin signaling, which is the most important signaling in regulation of this process. This hypothesis will be studied using the organoid model. The experiment needs to be performed to ensure the validity of the hypothesis; therefore no conclusion can be taken before that.

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