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Epigenetic Regulation by O-GlcNAc Alters Anti-Fibrotic Gene Expression in IPF Fibroblasts

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MULTIDISCIPLINARY BIOMEDICAL SCIENCE

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is an age-related chronic disease with a median survival period of 3-4 years [1]. Although FDA has approved two drugs that may slow the disease process, there is no effective treatment [2]. IPF is characterized by the excessive deposition of collagen, which leads to deformation of alveolar structure, loss of lung function, and ultimately death [3]. Anti-fibrotic genes such as cyclooxygenase-2 (COX-2) and Heme Oxygenase 1 (HMOX-1) are usually suppressed in the pathogenesis of IPF [4-6], while the mechanisms responsible for the repression of anti-fibrotic genes in IPF are incompletely understood. More evidence has shown that altered epigenetic marks such as histone methylation are involved in silencing anti-fibrosis genes [7]. H3K27Me3 is a key repressive histone mark that mediates gene repression, catalyzed by the methyltransferase enhancer of Zeste homolog 2 (EZH2) [8]. EZH2 expression is increased in lung fibrosis [9] and its levels/activity is regulated by the post-translational modifications, such as phosphorylation, acetylation, methylation, ubiquitination, sumoylation, and O-GlcNAcylation [10]. In this study, we investigate the effects of O-GlcNAcylation on anti-fibrotic gene expression via the EZH2-H3K27Me3 axis. Our results indicate that O-GlcNAc levels are increased in IPF lung tissue compared to control human lungs by IHC

staining and western blots. In primary human lung fibroblasts, inhibition of OGT (O-GlcNAc transferase) with the OGT inhibitor, OSMI-1, reduced O-GlcNAc levels and EZH2 protein expression as determined by Western blot analysis. In addition, we noticed the significantly increased expression of anti-fibrotic genes, COX-2 and HMOX-1, at the mRNA level in lung fibroblasts with OGT inhibition. Our ChIP assay confirmed that COX-2 and HMOX1 are regulated by H3K27Me3. This indicates that O-GlcNAc can regulate anti-fibrotic genes in lung fibrosis through EZH2-H3K27Me3 axis. Our results support a central role of O-GlcNAc for EZH2-mediated histone hypermethylation in anti-fibrotic genes epigenetic silencing in IPF.

Keywords: Epigenetics, IPF, O-GlcNAc, EZH2, H3K27Me3, Anti-fibrotic gene

DEDICATION

I dedicate my thesis work to my family, my mentor, and my friends. A special feeling of gratitude to my loving parents, whose words of encouragement and push for tenacity ring in my ears.

I also dedicate my mentor, Dr. Yan Y Sander, who have supported me throughout the process. Her sagacity, patience and optimistic spirit will encourage me to move forward in my future academic career.

I dedicate this work and give special thanks to my friends and colleagues for helping me develop my technology skills, revise mistakes, and master the leader dots.

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CHAPTER 1

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF)

Fibrosis is a process in which a normal organ or tissue is damaged beyond its ability to repair itself, and the damaged area is transformed into fibrotic tissue composed of extracellular matrix, including collagen and fibronectin [11]. Usually, fibroblasts participate in the tissue repair process, secreting extracellular matrix and promoting wound contraction. When the repair process is dysregulated, excess tissue is deposited and forms permanent scar tissue that occurs as a pathological process [12]. Fibrosis inhibits or interferes with normal tissue structure and function, leading to end-stage organ failure and death in many chronic diseases [13]. Fibrotic diseases attribute up to 45% mortality in the western world. A rare fibrotic diseases is idiopathic pulmonary fibrosis (IPF), which is part of a family of more than 200 lung diseases with similar symptoms, including all those that exhibit chronic inflammation or scarring in the lungs [14].

IPF is an age-related chronic disease of unknown etiology characterized by an abnormal excess of collagen that results in structural deformation of the alveoli, loss of lung function, and ultimately death [15]. There are more than 5 million IPF patients worldwide, with an average survival rate of 3-4 years, and there is no effective treatment with an increasing rate of 13-20 per 100,000 people per year [16]. Currently the FDA has only approved two drugs to slow the progression of the disease, which only delay the

progress of the disease, and new drugs are still needed to prevent or reverse this deadly disease [17].

The lack of therapeutic drugs is mainly due to the unclear pathogenesis of IPF [18]. The pathogenetic process of IPF has three stages: 1) Initiation stage: oxidative stress caused by various reasons acts as the initiation factor of IPF, including aging, smoking, virus infection, air pollution, etc [19]; 2) Advanced stage: alveolar cell, immune cell activation, secretion of various inflammatory factors, and injury of epithelium, endothelium, interstitium, collagen tissue and basement membrane [20]; and 3) Final stage: Type II alveolar epithelial cells (AEC II) proliferate and differentiate into type I alveolar epithelial cells (AEC I) to remediate the damage. Pulmonary fibroblasts are also activated, meanwhile, the expression of pro-fibrotic genes (COL1A1, COL3A1, α-SMA, etc) is increased, and the expression of anti-fibrotic genes (COX-2, HMOX-1, MMP1, etc) is decreased, which promotes fibroblasts to differentiate into myofibroblasts (FMT). Ultimately, these changes result in excessive extracellular matrix deposition, destruction of lung structure, and chronic respiratory failure [21]. Fibroblasts [22], fibrocytes [23], alveolar epithelial cells [24], immune cells [25], and myofibroblasts cells are all effector cells in pulmonary fibrosis with myofibroblasts being the ultimate ones [26, 27], causing abnormal extracellular matrix deposition, reduce lung compliance, aggravating epithelial injury and inflammation [11].

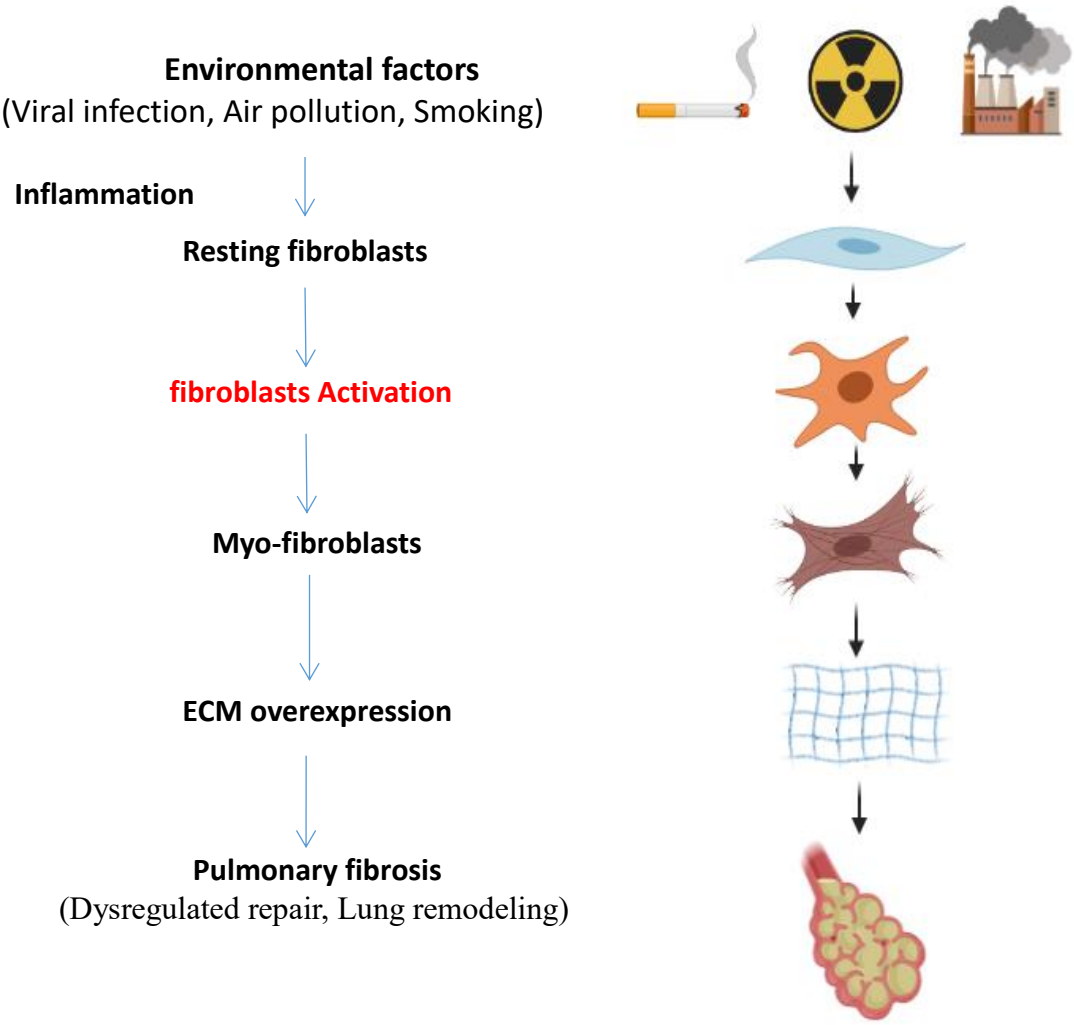

Figure 1. **Fibroblast activation in the pathogenesis of IPF.**

Fibroblasts can be activated to differentiate into myofibroblasts [28] (Figure. 1). Therefore, blocking their activation to inhibit fibrogenesis could be essential to improve fibrosis or promote resolution. Fibroblast activation can occur through several different mechanisms: stimulation of growth factors (such as TGF-beta), accumulation of ECM proteins, dynamic communication between cells, and changes in the physical and chemical

environment [29, 30]. Emerging studies have also shown that epigenetic remodeling is critical in regulating fibroblast activation [31-33].

Epigenetics

Epigenetics regulates gene expression without involving alterations in the DNA sequence [34]. Classical epigenetic mechanisms are categorized into three parts: DNA methylation, histone modification, and RNA interference (RNAi) (Figure. 2) [35]. DNA methylation is typically identified as the covalent bonding of a methyl group at the cytosine 5' carbon site of CpG island in the genome under the activity of DNA methylation transferase [36]. Previous studies have shown that DNA methylation can control gene expression by altering chromatin structure, conformation and stability of DNA and the association between DNA and transcriptional factors [37]. The N-terminus of histones can be subjected to a variety of modifications, including acetylation, methylation, phosphorylation, ubiquitination, ADP ribosylation, etc., all of which affect the transcriptional activity of genes [38]. RNA interference refers to gene silencing caused by specific degradation of homologous mRNA [39].

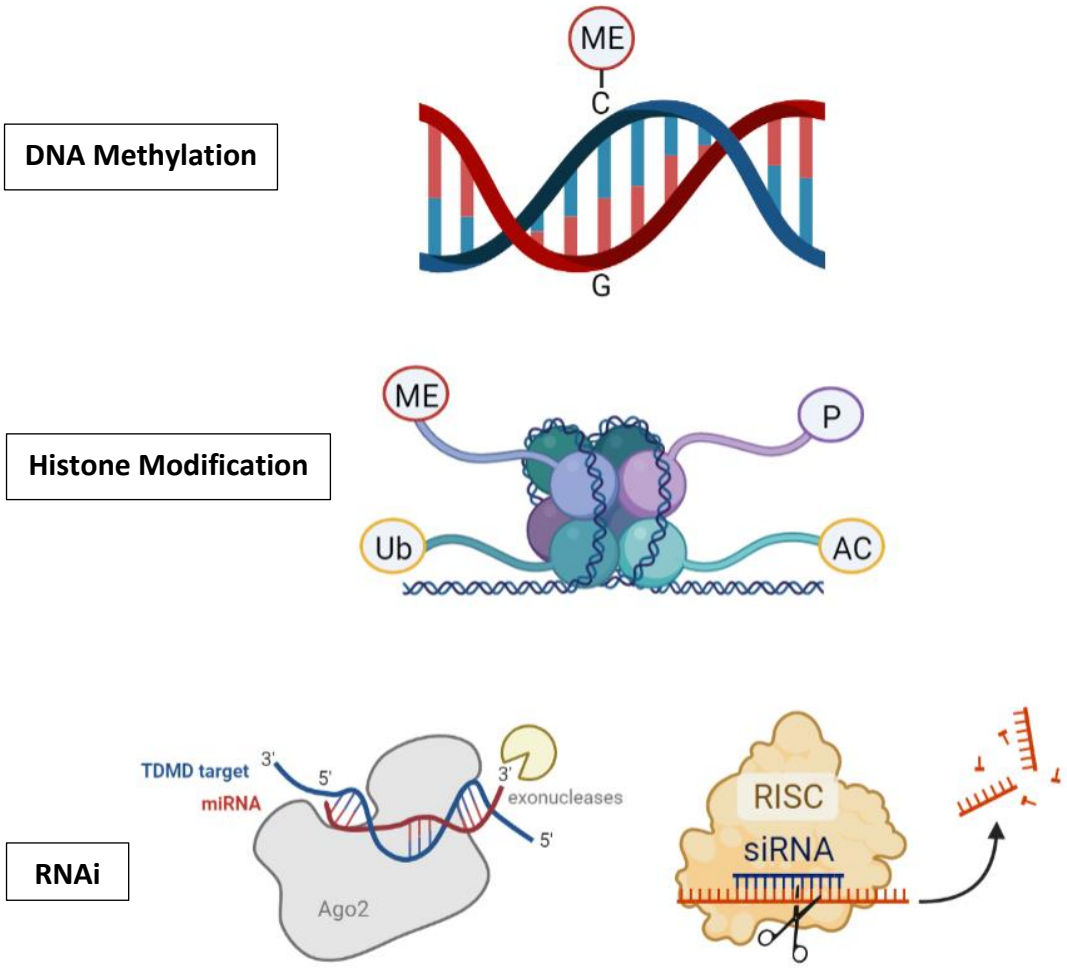

Figure 2. The three main areas of Epigenetics. The three main epigenetic alteration mechanisms are DNA methylation, histone modifications (acetylation, methylation, phosphorylation, etc.), and RNA interference. These three processes are distinct but are interrelated and control gene expression.

Epigenetic alterations caused by environmental exposure regulating chromatin accessibility is a key mechanism that mediates environmental effects on gene expression and disease [40]. Epigenetic changes have been shown to be involved in aging, cancer, diabetes, heart disease, mental illness, and other diseases [41]. Epigenetics also plays an important role in IPF [42]. Age, environment, and genetic factors are important in the pathogenesis of IPF [14]. The most commonly studied epigenetic modifications in fibrosis are DNA methylation and histone modifications, which are able to alter and influence

the availability of transcription factor binding to regulate gene transcription and cell function [43]. Previous studies have shown random epigenetic drifts of fibrotic-related DNA methylation with age may lead to disease development in susceptible individuals [44]. Studies have shown that reduced DNA methylation (hypomethylation) would increase the expression of pro-fibrotic gene, while hypermethylation would decrease anti-fibrotic gene expression [45, 46]. Our lab compared the level of total DNA methylation and DNA methyltransferase in IPF and normal lung tissues and found that the expression of DNA methyltransferase, DNMT3A (DNA Methyltransferase 3 Alpha) and DNMT3b (DNA Methyltransferase 3 Beta), was elevated in IPF patients, thus regulating the fibrosis-related genes through DNA methylation. For example, anti- fibrotic genes: THY-1 (Thy-1 or CD90 Cell Surface Antigen), ZNF467 (Zinc Finger Protein 467) and CLDN5 (Claudin 5) are decreased due to hypermethylation, while pro-fibrotic genes: Col3A1 (Collagen Type III Alpha 1 Chain), TP53INP1 (Tumor Protein P53 Inducible Nuclear Protein 1) and DDAH1 (Dimethylarginine Dimethylaminohydrolase 1) are elevated due to hypomethylation [47]. Another study by Yang et al., analyzed CpG islands in IPF patient samples by using comprehensive high-throughput arrays for relative methylation (CHARM) methodology. They did not only identify several genes previously associated with the disease but also identified a new gene, CASZ1 (Castor Zinc Finger 1), which is regulated by the methylation transferase EZH2. They also demonstrated that methylation changes on the shores of CpG island are more regulated than changes within the island [48]. Rabinovich et al. found that the DNA methylation in IPF samples overlapped with lung cancer samples by comparing the similarity of CpG methylation between ordinary lung tissue,

IPF and lung cancer. Their results suggest that methylation of tumor suppressor in IPF may be one of the causes of lung cancer with IPF susceptibilities [49].

Histone modification, on the other hand, not only regulates DNA methylation patterns but also directly participates in nucleosome interactions and influences chromatin structure [50]. Histone methylation and acetylation are the two most studied histone modifications. Histone acetylation is facilitated by histone acetyltransferase (HAT), which targets lysine (K) residues on the n-terminal histone tail, and histone deacetylation (HDAC) assists in the removal of these groups [51]. In IPF, histone methylation and acetylation play a critical role in regulating gene expression. Increased levels of H4K16Ac in aged mice with lung fibrosis by bleomycin-injury are thought to be involved in the activation of certain pro-fibrotic genes [51]. When H4K16Ac acetyltransferase is inhibited, the levels of pro-fibrosis genes such as α-SMA (α-smooth muscle actin), COL1A1 (collagen type I alpha 1 chain), and Nox4 (NADPH Oxidase 4) are significantly reduced [51]. Studies have shown that histone deacetylase inhibition can improve the resolution of lung fibrosis. An FDA-approved histone deacetylase inhibitor (HDACi), suberoylanilide hydroxamic acid (SAHA) down-regulates collagen 3A1 to ameliorate IPF [52]. SAHA also up-regulates the pro-apoptotic gene Bak (BCL2 Antagonist/Killer) and down-regulates the anti-apoptotic gene Bcl-XL (B-cell lymphoma-extra large) to promote fibroblast apoptosis in bleomycin-induced mice [52, 53]. Besides histone acetylation, repressive histone marks are also involved in the regulation of fibrosis-related genes [54]. For example, repressive histone marks H3K9me3 and H3K27Me3 are associated with promoters of the anti-fibrosis genes COX-2 (cyclooxygenase-2) and CXCL10 (C-X-C motif chemokine ligand 10) which are down-regulated in IPF. While inhibiting methyltransferases

such as EHMT2 (Euchromatic histone-lysine N-methyltransferase 2) and EZH2 (Enhancer of zeste homolog 2) could reactivate the expression of anti-fibrotic genes [55-57].

In addition to DNA methylation and histone modifications, other epigenetic marks are also studied in IPF, such as microRNAs (miRNAs) [58], LncRNA (Long noncoding RNAs) [59], and m6A (N6-methyladenosine, a type of RNA modification) [60]. Crosstalk between different types of marks help us integrating various epigenetic mechanisms in IPF [59]. Epigenetics provides a broader understanding of the pathogenesis and regulatory mechanisms of IPF, such as transcriptional, post-transcriptional and post-translational regulation. Epigenetic marks provide novel therapeutic targets for lung fibrosis.

EZH2-H3K27Me3 Axis

Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that belonged to the polycomb repressive complex 2 (PRC2) [61]. It is an evolutionary conserved subunit of PRC2, collaborating with other subunits such as SUZ12, EED, SET, etc, catalyzing histone H3K27Me3 to repress the target gene expression [62]. Independent to PRC2, EZH2 also acts as a co-activator for transcription factors activating target gene expression [63]. Maintaining the balance of EZH2 is essential for physiological processes and cellular functions such as proliferation, apoptosis, DNA repair, and senescence [64]. Interference with EZH2 homeostasis can lead to a variety of diseases such as cancer, neurodegenerative diseases, and fibrotic diseases [65-67]. In cancer, levels of EZH2 and H3K27Me3 are usually overexpressed and turn off tumor suppressor genes. For example, EZH2 inhibits the expression of tumor suppressor gene DLC1 (deleted in Liver Cancer 1)

through catalyzing tri-methylation of H3K27 at the promoter of DLC1 in liver, lung, breast, prostate, and ovarian cancer tissues [68]. EZH2 overexpression may also enhance chemotherapeutic resistance [69]. In small cell lung cancer, overexpressed EZH2 inhibits the expression of SLFN11, a factor associated with DNA repair, leading to chemotherapy resistance [70]. In lung fibrosis, inhibiting EHZ2-H3K27Me3 increases the expression of anti-fibrotic genes such as COX-2 and HMOX-1 [55, 71]. In addition, EZH2 also regulates innate immune responses in lung fibrosis via mediating the imbalance of macrophage polarization [72]. EZH2 is also responsible for the healthy development of embryos [73]. EZH2 mutation would cause a global redistribution of gene repressive markers leading to primary cancer [74]. Mutation of mesodermal EZH2 leads to the formation of ectopic smooth muscle in lung development and increases the risk of developing IPF, COPD, and other lung diseases[75, 76]. Thus, maintaining the homeostasis of EZH2 is vital. The stability of EZH2 can be regulated by RNA interference and Post-translational modifications, including phosphorylation, O-linked-N-Acetylglucosamine (O-GlcNAc modification or O-GlcNAcylation), acetylation, methylation, and ubiquitination, in which phosphorylation is usually responsible for disassembling EZH2 and O-GlcNAcylation is responsible for stabilizing EZH2 [77]. In our study, we investigated the role of O-GlcNAcylation in regulating the EZH2 and H3K27Me3 axis in IPF.

O-linked N-Acetylglucosamine (O-GlcNAc)

The O-GlcNAc is a post-translational modification that is catalytically added to serine and threonine residues of nuclear and cytoplasmic proteins. Early studies have not found protein-saccharide linkage until 1984, O-GlcNAc was first demonstrated in murine

lymphocytes [78]. In subsequent years, O-GlcNAc was shown to be present in the cytoplasm and subcellular organelles of rats, nuclear envelope, nuclear pore proteins, cytoskeletal protein of human erythrocytes, and polytene chromosomes in the salivary glands of *Drosophila* larvae [78]. So far, O-GlcNAc is widespread in eukaryotes and linked to metabolic and epigenetic homeostasis in physiology [79]. O-GlcNAcylation is a reversible and dynamic process regulated by two core enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) that are responsible for adding and removing O-GlcNAc modification onto the serine and threonine residues (Figure. 3) [80]. In addition, there is also another recently discovered O-GlcNAc transferase called EOGT that transfer 'GlcNAc' to Ser or Thr in secreted and membrane proteins [81]. However, the EOGT is not present in the nucleus and cytoplasm and will not be a focus here.

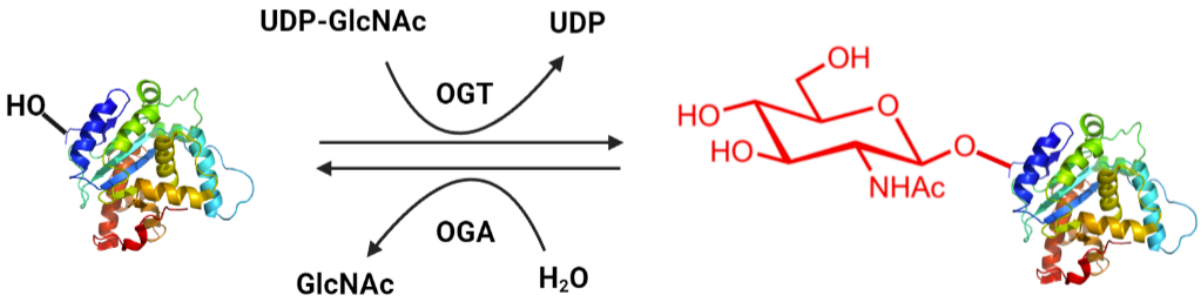

Figure 3: OGT and OGA. The balance of O-GlcNAcylation on serine and threonine residues is controlled by OGT and OGA.

Cumulative studies have indicated O-GlcNAcylation is not only a product of nutrient metabolism [82], but also acts a nutrient and stress sensor regulating cellular stress [83], signal transduction [84], transcription [85], and translation [86]. Dysregulation of O-GlcNAcylation of various protein substrates is associated with many chronic diseases, including fibrotic diseases [87], cardiovascular diseases [88], neurodegenerative diseases

[89], diabetes [90], and cancer [91]. Thus, investigating the role of O-GlcNAc in different diseases not only increase understanding of pathogenesis but provide new perspectives of treatments. In this case, we focused on the epigenetic role of O-GlcNAcylation in IPF.

Role of O-GlcNAcylation in Epigenetics

The epigenetic role of O-GlcNAcylation has been well studied. In epigenetics, a class of enzymes that regulate gene expression are often categorized as writers, readers, and erasers, in which writers install modifications on DNA and histones, readers identify and interpret these modifications, and erasers remove these modifications [92]. (Figure 4)

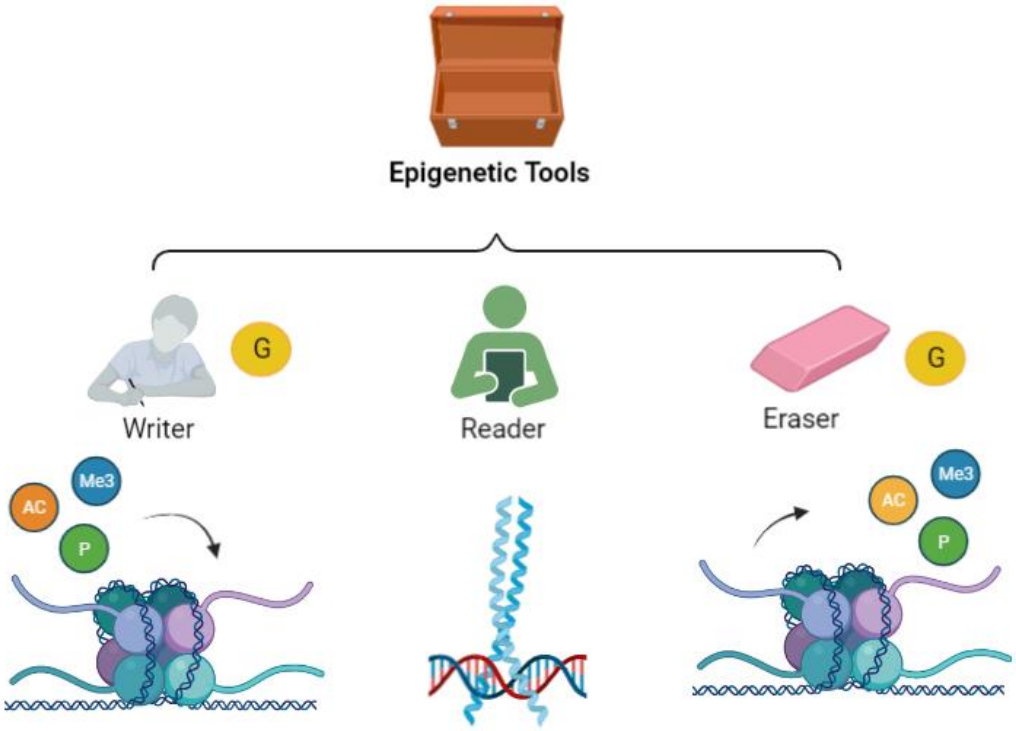

Figure 4: **Example of functions of Writers, Readers, and Erasers in epigenetics.** O-GlcNAc regulates writer and eraser in epigenetics.

OGT and OGA are identified on writer and eraser enzymes in epigenetics [93]. As writers, all core histones (H2A, H2B, H3, and H4) on nucleosomes can be modified by O-

GlcNAc and participate in the activation or suppression of gene transcription, stabilizing chromatin structure, and maintaining genomic stability [94]. OGT catalyzes the addition of O-GlcNAc to the ten-eleven translocation (TET) family of dioxygenases, an eraser, and an important demethylase removing methyl group. O-GlcNAc leads to the nuclear export of TET3, reducing its demethylated activity by depleting it from the nucleus [95]. Similarly, O-GlcNAc stabilizes methyltransferase EZH2, a “Writer”, in different sites to enhance the activity of methyltransferase [96]. O-GlcNAc also affects other histone modifications to regulate transcription, such as histone acetylation [97] and monobituation [98]. Like other modifications, O-GlcNAc is widely involved in physiological and/or pathological activities such as apoptosis, the cell cycle, and stress responses [99]. Impaired regulation of O-GlcNAc has been shown to involve in many pathologies including Alzheimer's disease, cancer, diabetes, and neurodegenerative disorders. (Figure 5)

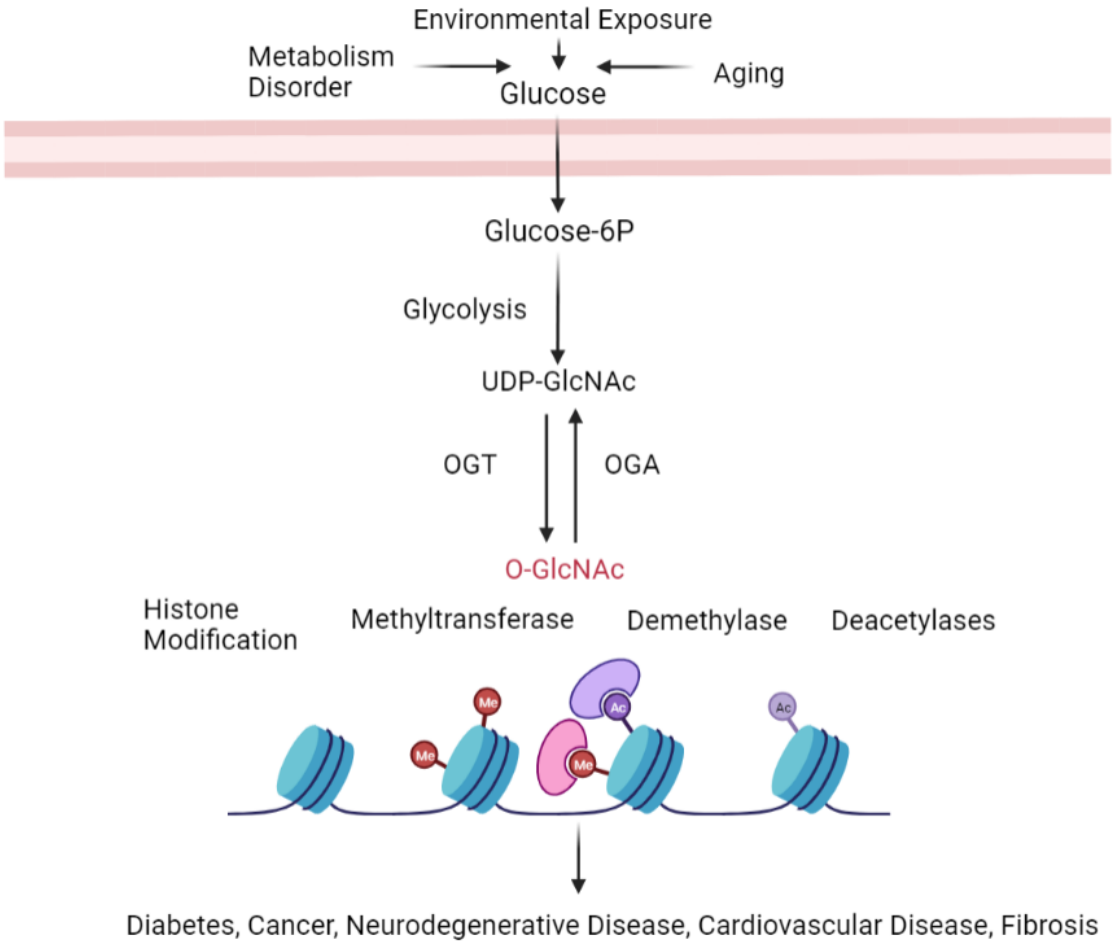

Figure 5: O-GlcNAc is an important product in hexosamine biosynthetic pathway regulating multiple chronic diseases via stabilizing epigenetic modification and related enzymes.

Role of O-GlcNAcylation in Fibrosis and Cancer

Previous studies indicate that OGT plays a regulatory role in many different types of fibrosis, for example, OGT protects hepatocytes against necroptosis and liver fibrosis by decreasing the expression and stability of RIPK3 (receptor-interacting protein kinase 3) which is considered as a key mediators of necroptosis [87], In renal interstitial fibrosis, O-GlcNAc is up-regulated in UUO rats model, and O-GlcNAcylation is proved to promote in the Epithelial-mesenchymal transition (EMT) of HK2 cells by inhibiting

RAF1 ubiquitination. In pulmonary fibrosis, the level of OGT increased and showed a characteristic of inducing fibrosis by elevating the expression of profibrotic genes such as A-SMA and Collagen [100, 101]. Thus, maintaining an optimal level of O-GlcNAc in different tissues and understanding the mechanism of O-GlcNAc regulation is crucial. In lung cancer, O-GlcNAcylation significantly drives the non-adherent growth of lung cancer cells and enhances malignancy by increasing the level of OGT [102]. Similarly, OGT also promotes proliferation of pulmonary arterial smooth muscle cell (PASMC) in idiopathic pulmonary arterial hypertension (IPAH) [103]. Interestingly, there is a positive correlation between OGT and OGA levels in lung adenocarcinoma. This tight and coordinated regulation maintains the homeostasis of O-GlcNAcylation in cancer [104]. Although many studies have shown that O-GlcNAc plays an important regulatory role in lung diseases, the role of OGT and O-GlcNAc in IPF has not been studied.

Epigenetic regulation of OGT in IPF

Previous evidence has shown that altered epigenetic modification such as histone hypermethylation promotes the silence of anti-fibrotic genes [7]. H3K27Me3 is a key repressive histone mark that mediates gene repression, which is catalyzed by the EZH2 [8]. EZH2 expression is increased in lung fibrosis [9], and its levels and activity are regulated via post-translational modification, such as phosphorylation, O-GlcNAcylation, acetylation, methylation and ubiquitination [10]. In this study, we investigate the effects of O-GlcNAcylation on anti-fibrotic genes expression via the EZH2-H3K27Me3 axis. This research will deepen our knowledge studying interactions between metabolism and epigenetics in IPF; and will aid in identifying targets for novel therapies for the treatment of

IPF or other fibrotic-related diseases. Our preliminary data showed O-GlcNAc levels were increased in IPF lung tissue compared to control non-IPF human lungs by IHC staining and western blots. In normal and IPF lung fibroblasts, inhibition of OGT with OGT inhibitor, OSMI-1, reduced O-GlcNAc levels, and reduced the expression of EZH2, which is the methyltransferase of the repressive histone mark H3K27Me3 by western blot analysis. This implies that O-GlcNAc likely regulates the expression of anti-fibrotic genes through EZH2-H3K27Me3 axis in lung fibrosis.

Hypothesis

In this thesis, we hypothesize that anti-fibrotic genes are regulated through the OGT-EZH2-H3K27Me3 axis *in vivo* and *in vitro*. We proposed to examine some anti-fibrotic genes that OGT regulates by RT-PCR/western-blot and to confirm the association of anti-fibrotic genes via the EZH2-H3K27Me3 axis by ChIP assays. In addition, an animal model of bleomycin-induced lung fibrosis in mice as an *in vivo* model was used to evaluate the effects of targeting OGT as a therapeutic method for lung fibrosis.

CHAPTER 2

MATERIALS &METHODS

Cell Culture and Treatments

A normal lung fibroblast cell line, IMR90 were bought from ATCC. Human primary IPF and non-IPF lung fibroblasts were derived from de-identified lung tissues from the University of Alabama at Birmingham (UAB) Tissue Procurement Facility, which was approved by the UAB Institutional Review Board. Cells were cultured in DMEM with 2 mM L-Glutamine and 10% FBS (full medium), when cells were 80% confluent, O-GlcNAc transferase (OGT) inhibitor (OSMI-1, 25μm), or OSMI-4 (5μm) or O-GlcNAcase (OGA), the enzyme removing O-GlcNAc, inhibitor (Thiamet G, 25nM) were added directly in fresh full medium for 48h, then the cells were collected for various assays.

RNA extraction, qRT-PCR

RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA), and transcribed into cDNA with a cDNA synthesis kit (Takara Bio, Mountain View, CA). Real-time RT-PCR was performed in triplicate and normalized to β-actin by using the $2^{-\Delta\Delta CT}$ method. mRNA levels of COX-2 and HMOX-1 were examined by quantitative real-time PCR with primers. COX-2: 5'-CCGGGTACAATCGCACTTAT-3' and 5'-

GGCGCTCAGCCATACAG-3'; HMOX-1: 5'-CCAGGCAGAGAATGCTGAG-TTC-3' and 5'-AAGACTGGGCTCTCCTTGTTGC-3'

Western Blots

Whole cell lysates were collected and quantified with a Micro BCA Protein Assay kit (Thermo Scientific). Nuclear extracts were collected with the EpiQuick Nuclear Extraction kit (Epigentek, Farmingdale, NY), then quantified by BCA assay. Lysates or nuclear extracts were subjected to SDS-PAGE and western immunoblotting (WB). Immunoblots were imaged with an Amersham Biosciences 600 Imager (GE Healthcare). Densitometry analysis was done using Image J software. Cell lysates were subjected to SDS-PAGE gels, and probed with anti-EZH2 (Cell signaling, Cat# 5246), H3K27Me3 (Cell Signaling, Cat# 9733), COX-2 (Abcam, Cat# ab15191), HMOX-1 (Cell signaling, Cat# 5853), antibodies; signals were detected using an enhanced chemiluminescence system.

Chromatin Immunoprecipitation assay (ChIP)

Chromatin immunoprecipitation (ChIP) assays were performed as per the manufacturer's protocol (ab500, Abcam, Cambridge, MA). An antibody against EZH2 (Cell signaling, Cat# 5246) and H3K27Me3 (Cell signaling, Cat# 9733), were used to pull down the associated DNA. ChIP-DNA were amplified by real-time PCR with primers of HMOX-1 and COX-2, using SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, CA). Results were normalized to input DNA. DNA was immunoprecipitated using

antibodies for EZH2, or H3K27Me3, while the association between EZH2 or H3K27Me3 with HMOX1 and the COX-2 promoter region was determined by realtime PCR.

Immunohistochemistry

Sections from paraffin-embedded lung tissues were immunostained with EZH2 (Cell signaling, Cat# 5246) and H3K27Me3 (Cell signaling, Cat# 9733). Briefly, slides from paraffin block were rehydrated with xylene, ethanol, and water. After heat induced epitope retrieval in Thermo citrate buffer (Thermo Scientific, Cheshire, WA), slides were immunostained according to the Dako Rabbit Envision System (Dako, Carpinteria, CA) protocol. Images were obtained with a Keyence BZ-X700 All-in-one microscope (Itasca, IL).

Aged mice lung fibrosis model

All animal studies were performed in accordance with UAB Institutional Animal Care and Use Committee approved protocols. 18 month old C57BL/6 mice were subjected to oropharyngeal administration of saline or bleomycin at 1.25U/kg to induce lung fibrosis. 3 weeks after injury, OGT siRNA was given nasally every another day at 25mg/kg to the bleomycin injured and siRNA NT control mice. All mice lung tissues were collected at 6-week post injury for various assays.

Statistical Analysis

Data were presented as the mean $\pm$ standard deviation (SD). All data were statistically analyzed with GraphPad Prism (La Jolla, CA). T-test was used to compare two

groups. One-way ANOVA was used to compare between multiple groups; comparison between two groups were done using Student's paired t test. A p value <0.05 was used to determine statistical significance.

CHAPTER 3

RESULTS

EZH2 is downregulated in IPF and IMR90 fibroblasts by OGT inhibition

Increased OGT is found in IPF lung compared to the non-IPF lung [53], and an up-regulated EZH2 in IPF fibroblasts or lung tissues of mice with bleomycin injury [9]. To investigate the role of O-GlcNAc in IPF and whether OGT regulates EZH2 gene expression, we assessed EZH2 levels in IMR90 and IPF fibroblasts with and without OGT inhibitor for 48 hours. Lower levels of EZH2 were noticed with OGT inhibition in both IMR90 and IPF fibroblasts. However, the OGA inhibitor, Thiamet-G (TMG), had no significant effect on the levels of EZH2 (Figure 6A, B). In addition, since TGF-β 1 is a crucial profibrotic cytokine inducing fibroblasts to myofibroblasts transition (FMT), increased EZH2 level is found in IMR90 fibroblasts with TGF-β 1 treatment, and OGT inhibition blocks TGF-β induced EZH2 expression (Figure 6C). These data indicate that OGT inhibition can reduce increased EZH2 in both IPF fibroblasts and TGF-β 1-induced myofibroblasts.

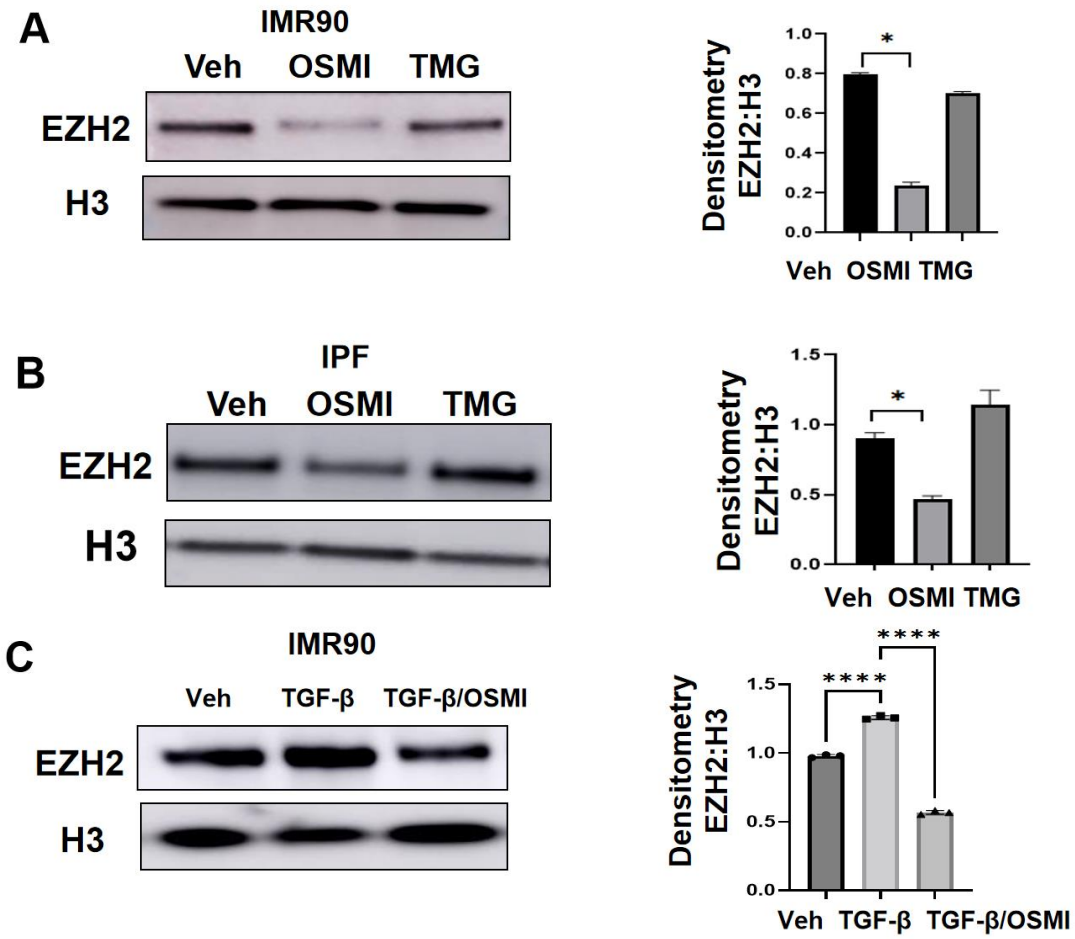

Figure 6: EZH2 is downregulated in IPF and IMR90 fibroblasts by OGT inhibition. The effect of OGT inhibition on EZH2 expression in lung fibroblasts of IMR90 (A), IPF (B) and TGF-B treated IMR90 fibroblasts (C) were carried out with OGT inhibitor OSMI-1 (25μM), or with OGA inhibitor TMG (25nM) for 48h. See detailed protocol in the Methods section. Nuclear extracts were subjected to WB to examine the EZH2 expression levels, and H3 is the loading control. *p<0.05 compared to vehicle control or as indicated.

Increased expression of the anti-fibrotic genes HMOX-1 & COX-2 with OGT inhibition in IPF and non-IPF fibroblasts

Anti-fibrotic genes have anti-inflammatory and antioxidant properties, playing an important role in IPF [105]. We hypothesized that the expression of related anti-fibrotic genes is regulated by O-GlcNAc through the EZH2-H3K27Me3 axis. We then analyzed

the expression of two anti-fibrotic genes, HMOX-1, and COX-2, with or without OGT inhibition. We observed that mRNA and protein levels expression (Figure 7E, F, G) of anti-fibrotic genes HMOX-1 and COX-2 are increased with OSMI treatment in IPF (Figure 7A, B), and with OSMI added TGF-β1 treated non-IPF fibroblasts (Figure 7C, D). Similarly, we did not observe significant changes in these genes' expression by the OGA inhibitor, TMG. This data suggests that OGT inhibition can regulate the expression of the anti-fibrotic genes, HMOX-1, and COX-2.

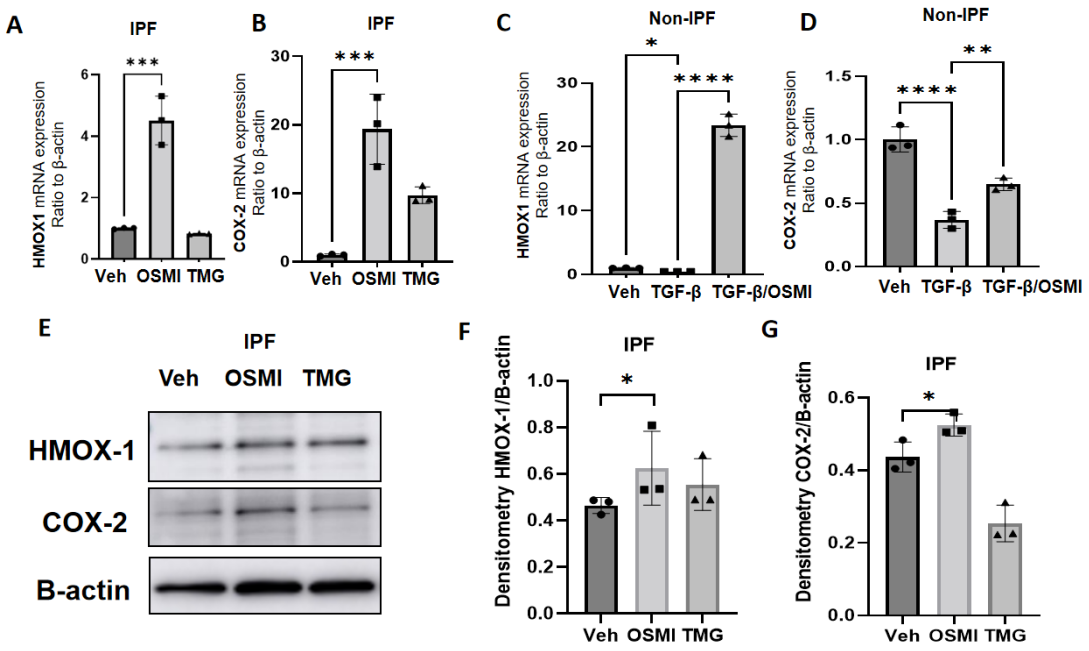

Figure 7. Increased expression of the anti-fibrotic genes HMOX-1 & COX-2 with OGT inhibition in IPF and non-IPF fibroblasts mRNA. Expression of anti-fibrotic genes HMOX-1 (A) & COX-2 (B) with OSMI-1 (25μM) or TMG (25nM) treatment for 48h in IPF. mRNA expression of HMOX-1 (C) & COX-2 (D) in TGF-β (5μg/ml) induced Non-IPF fibroblasts with OSMI-1 (25μM) or TMG (25nM) treatment. E: Level of HMOX-1 and COX-2 under OSMI treatment. See detailed protocol in the Methods section. Whole cell lysates were used for WB and analyzed by densitometry of HMOX-1 (F) and COX-2 (G) protein level ratio to β -actin *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.

Repressive histone H3K27me3 is enriched at the HMOX-1 and COX-2 promoter in IPF due to OGT or EZH2 inhibition

To verify whether OGT inhibition regulates the expression of anti-fibrotic genes through the EZH2-H3K27me3 axis, we used a ChIP assay to detect the recruitment of H3K27me3 to the promoter of anti-fibrotic genes after OGT and EZH2 inhibition (by using OSMI and EPZ6348). First, we found that the expression of HMOX-1 and COX-2 increased after EZH2 inhibition (Figure 8A, B), which is similar to OGT inhibition, suggesting that OGT and EZH2 have similar effects and may modulate the regulation of these genes through the same pathway. Then, ChIP assay results showed a depleted association with H3K27me3 at COX-2 and HMOX-1's promoter regions (Figure 8C, D). Since H3K27me3 is a gene inhibition marker, more enrichment of it leads to decreased gene expression, which is consistent with the mRNA data above.

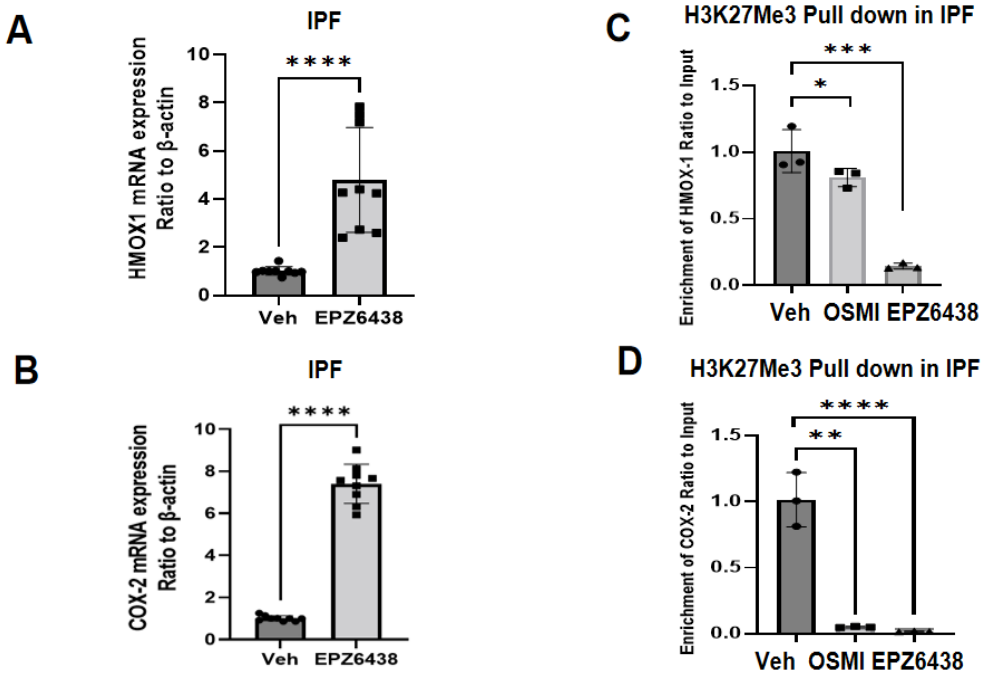

Figure 8. **Histone H3K27me3 is enriched at the HMOX-1 and COX-2 promoter in IPF due to OGT or EZH2 inhibition mRNA.** Expression of HMOX1 (A) and COX-2 (B) with EZH2 inhibitor (EPZ6438; 5 μM). EZH2 inhibitor (EPZ6438) and OSMI decrease the anti-fibrotic genes, HMOX-1 (C) and COX-2 (D) promoter region association with H3K27Me3. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.

OGT inhibition blocked the down regulation of HMOX-1 and COX-2 by TGF-β through reduced association of H3K27Me3 at their promoter regions

Next, we tested whether OGT inhibition regulates anti-fibrotic genes in TGF-β1 treated non-IPF fibroblasts through the EZH2-H3K27Me3 axis. It is noticed that the enrichment of H3K27Me3 (Figure 9 A, B) and EZH2 (Figure 9 C, D) on HMOX-1 and COX-2 promoters were increased 48h after the treatment with TGF-β1, which was corresponding to the decreased mRNA expression (Figure 7 C, D). Conversely, OSMI decreased EZH2 and H3K27Me3 enrichment significantly at the promoter of HMOX-1 and COX-2. This data showed that TGF-β1 reduced the expression of anti-fibrosis by increasing the enrichment of EHZ2 and H3K27Me3 in the FMT process, while OGT inhibition blocked the recruitment of EZH2 and H3K27Me3 at the promoter region of HMOX-1 and Cox-2 upon TGF-β1 stimulation, resulting increased mRNA compared to TGF-β only (Figure 7 C, D).

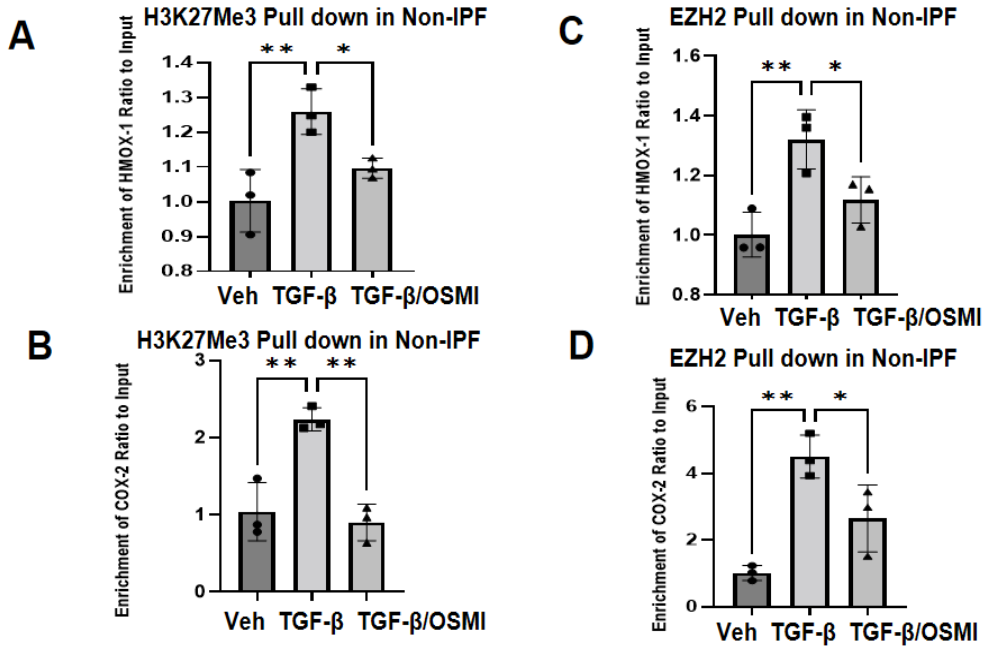

Figure 9. OGT inhibition blocked the down regulation of HMOX-1 and COX-2 by TGF-β through reduced association of H3K27Me3 at their promoter regions. H3K27me3 (A, B) and EZH2 (C, D) associations are increased at the promoter region of HMOX-1 and COX-2 in TGF- β induced non-IPF fibroblasts and depleted with OSMI/TGF- β treatment. *P ⩽ 0.05; **P ⩽ 0.01.

OGT inhibition by siRNA OGT (nasal delivery) reduces EZH2 in the lung tissues of aged mice with persistent lung fibrosis

To test the effect of OGT inhibition *in vivo*, we established a bleomycin lung fibrosis model in 18-month old mice, which is the most common model to mimic pulmonary fibrosis and investigate therapies for IPF [106]. Mice were subjected to bleomycin to induce lung injury, after three weeks post-injury, they were treated with OGT siRNA for another three-week via nasal delivery, and their lung tissues were collected for Immunohistochemistry (IHC) assay and protein analysis (Figure 10A). EZH2 levels are reduced in the murine lung tissues in the group with bleomycin injury and siOGT treatment compared to those bleomycin only determined by IHC staining (Figure 10B for representative figures) and Western blots (Figure 10C). In addition, whole lung lysates showed

increased HO-1 protein levels by Western blot analysis in the siOGT-treated group in comparison with induced expression in bleomycin-injured mice (Figure 10D, E). However, COX-2 protein levels were decreased by siOGT compared to the bleomycin group (Figure 10D, F).

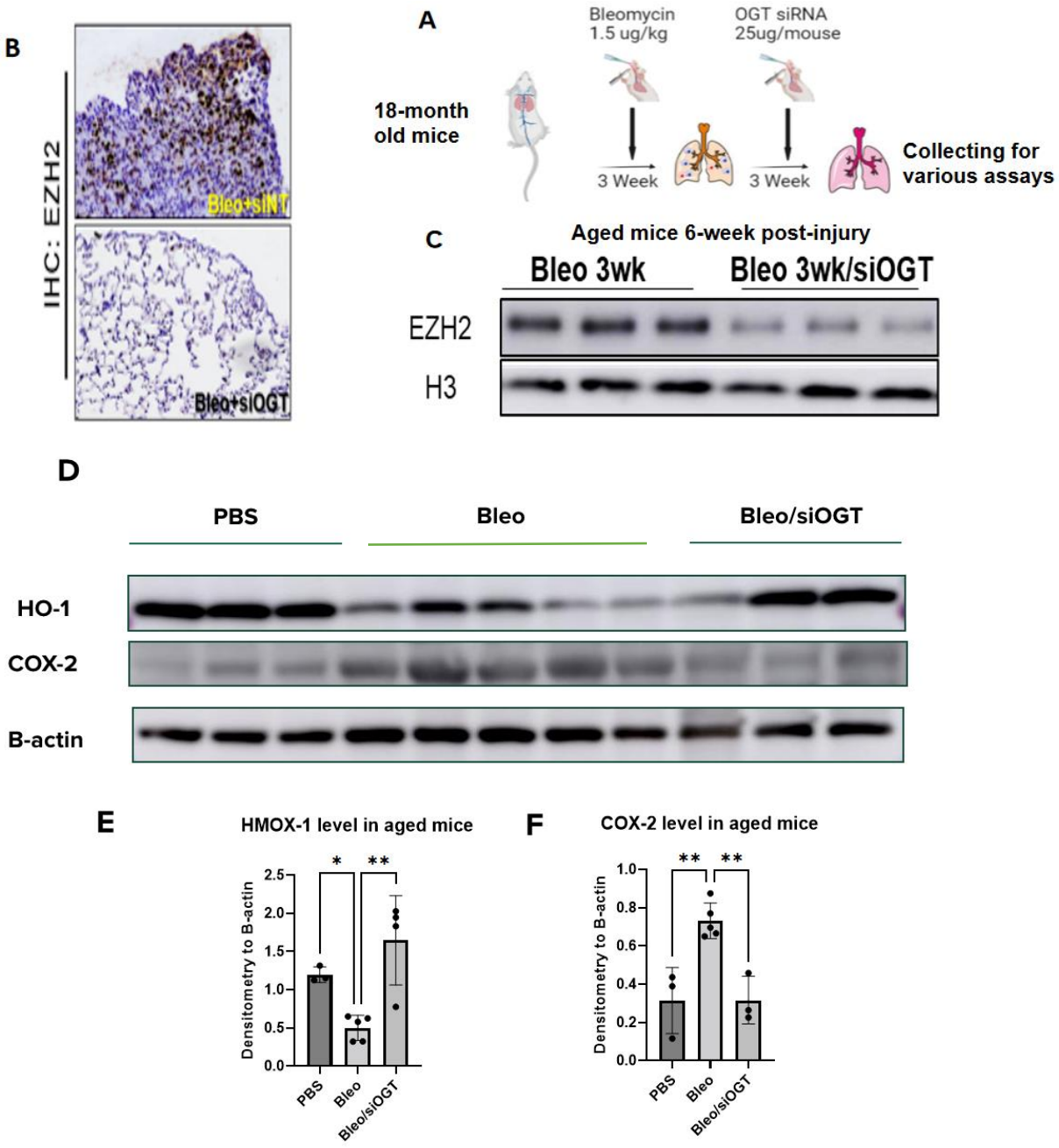

Figure 10. OGT inhibition by siRNA OGT (nasal delivery) reduces EZH2 in the lung tissues of aged mice with persistent lung fibrosis. A. 18- month old (aged) mice were injured with bleomycin. A. 18- month old (aged) mice were injured with bleomycin. At 3 weeks post-injury, the mice were administrated with OGT siRNA for treatment for 3 weeks. All mice were sacrificed at 6-week after injury, and the lung tissues were collected for various assays. B. EZH2 immunohistochemistry in the mice treated with Bleomycin and non-targeting (NT) control, or with siRNA OGT. C. EZH2 level were examined in the lung tissues with bleomycin or bleomycin with siRNA OGT treatment. D. Level of HMOX-1 and COX-2 in the lung tissues with bleomycin or bleomycin with siRNA. Densitometry of HMOX-1 (E) and COX-2 (F).

CHAPTER 4

DISCUSSION

The development of IPF is associated with the downregulation of anti-fibrotic genes due to epigenetic dysregulation, in which histone methylation plays a major role in the regulation of gene expression and inhibition [59]. Stability of methyltransferases are maintained by post-translational modifications including the O-GlcNAc modification. Studies indicate that thousands of proteins are modified by O-GlcNAc [107]. Although some progress has been made, the function of O-GlcNAcylation in epigenetics is still not fully clear [108]. Our study investigated the epigenetic role of O-GlcNAc in IPF. The data indicate that the OGT inhibitor, OSMI, increases transcription of anti-fibrotic genes including HMOX1 and COX-2 likely through decreased expression of the methyltransferase EZH2 to decrease histone modification H3K27Me3. The decreased recruitment of repressive mark H3K27Me3 at the promoter region resulted in increased expression of the mRNA of these genes. HMOX-1 is a type of Heme oxygenase (HO) degrading heme to biliverdin, free iron, and carbon monoxide (CO). Biliverdin is rapidly reduced to bilirubin by the activity of the biliverdin reductase, and free iron is released by ferritin. At physiological concentrations, bilirubin and free iron are considered as antioxidant, and CO has strong cytoprotective, anti-inflammatory, antioxidant, and bactericidal properties [109]. There are three isoforms of HO that human may encode: HMOX-1, HMOX-2, and

HMOX-3, of which only HMOX-1 is the stress-induced and is considered to be an oxidative stress reactive protein [110]. In the pathogenesis of pulmonary fibrosis oxidant-antioxidant balance is disrupted. HMOX-1 act as a key defender against oxidative stress playing an important role in fibrotic lung disease [111]. Similar to our findings, HO-1 expression was down-regulated in IPF alveolar macrophages, possibly due to the partial loss of antioxidant capacity in IPF patients [111]. HO-1 is also significantly expressed in mice model of pulmonary fibrosis. A study showed that adenovirus-mediated HO-1 overexpression has protective effects by down-regulating pro-inflammatory cytokines [112]. In contrast, transforming growth factor -β1, an important pro-fibrotic cytokine, down-regulated HO-1 in mice lungs [113], which is also confirmed in this study. Similarly, we demonstrated that another anti-fibrotic gene COX-2 is up-regulated by OGT inhibition in IPF fibroblasts. COX-2 is regarded as an anti-fibrotic gene because of its main anti-fibrotic metabolite prostaglandin E2 (PGE2) [114]. Inhibition of COX-2 and PGE 2 expression lead to the activation of fibroblasts and excessive accumulation of collagen in pulmonary fibrosis [115]. COX-2 expression in lung fibroblasts of IPF patients is epigenetically silenced and can be remediated by epigenetic inhibitors such as LBH589 (panobinostat, a pan-HDAC inhibitor), BIX02189 (a G9a inhibitor), and 3-deazaneplanocin A (DZNep, an EZH2 inhibitor) [55, 116].

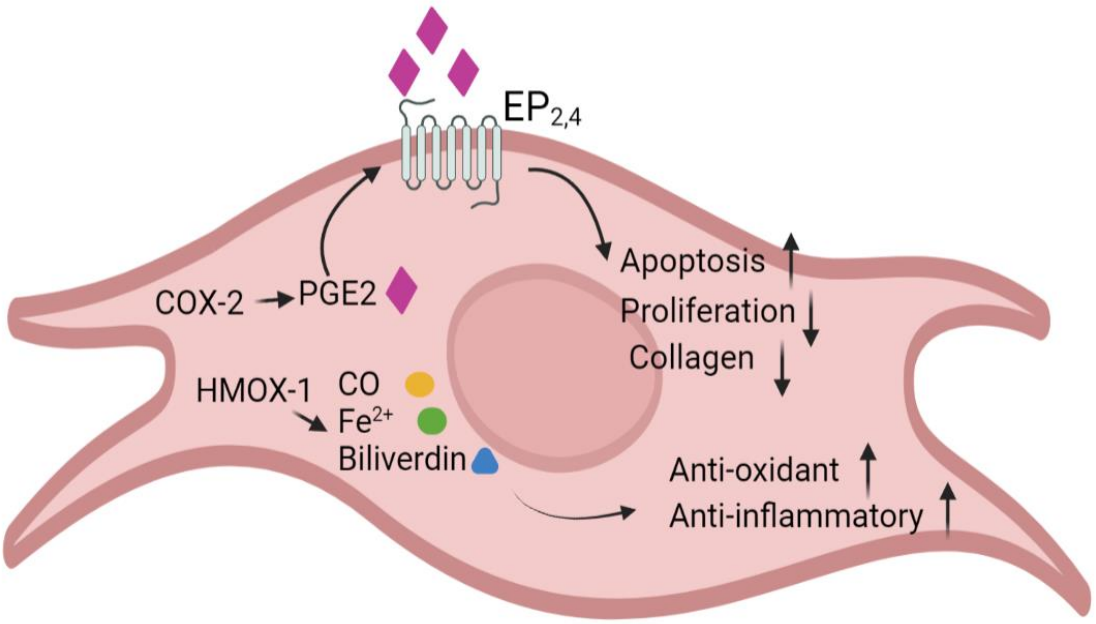

Figure 11. Enzymatic activity of COX-2 and HMOX-1. COX-2 enzymatic activity produces PGE2 to decrease the deposition of collagen and proliferation and promote apoptosis in fibroblasts. HMOX-1 enzymatic activity generates biliverdin and releases carbon monoxide (CO) and Fe^{2+} to increase the anti-oxidant and anti-inflammatory ability in fibroblasts.

In this study, we demonstrated that OGT inhibition restored COX-2 levels by reducing the level of EZH2 in IPF fibroblasts. Interestingly, COX-2 was not down-regulated in bleomycin-induced mouse models but was significantly increased, contrary to the results in fibroblasts from IPF patients. This may be explained by several reasons. First, it is the compensatory effect that mice show upregulation of COX-2 after bleomycin injury to reduce inflammation [114]. Second, it is also likely that we used whole lung tissue, which has multiple cell types other than fibroblasts. Different from IPF which is characterized by advanced fibrosis, the bleomycin mouse model is caused by acute lung injury that leads to early fibrosis, resulting in a large amount of fibroblast proliferation and collagen production in a short period of time. This would lead to the induced stress upregu-

lation of COX-2. In liver fibrosis, inflammation would be also triggered by overexpression of COX-2 [117]. Thus, the balance of COX-2 may have an important role in regulating fibrogenesis. To confirm that the increase of COX-2 was due to early response to inflammatory stress, lung tissues could be collected and analyzed in batches from the first and second weeks, at the inflammation stage. To eliminate interference from other cells, investigating COX-2 levels in isolated fibroblasts cells from bleomycin-induced mice would be an alternative way in the future study.

As a dynamic post-translational modification, O-GlcNAc has been involved in a variety of epigenetic regulatory mechanisms [118]. OGT and OGA are the key enzymes in the regulation of O-GlcNAcylation [119]. Previous studies in breast cancer have shown that the stability of the methyltransferase EZH2 is regulated by OGT [120], which has not been explored in IPF. In this study, we verified that OGT inhibition decreases the binding of EZH2 and H3K27Me3 to the anti-fibrotic genes' promoter. However, this study did not prove whether OGT directly binds EHZ2 in IPF. To confirm this, co-immunoprecipitation of OGT and EZH2 is expected to be utilized in the future experiment, and GC/MS could be used to determine the binding site of OGT on EZH2. Another interesting observation is, the OGA inhibitor, Thiamet-G, had no significant effects on the global level of EZH2, which was consistent with previous studies, probably due to a compensatory effect or positive feedback mechanism between OGT and OGA [104, 120]. OGT transfers O-GlcNAc onto EZH2 and stabilizes it, whereas ubiquitination or phosphorylation promotes EZH2 decomposition, and their equilibrium and crosstalk maintain the level and activity of EZH2 [77]. Due to the different locations and number of recognition sites for O-GlcNAcylation and phosphorylation on EZH2, even if O-GlcNAcylation

is not removed by Thiamet-G, EZH2 can be recognized and decomposed by phosphorylation or ubiquitination at other sites. (Figure. 12) However, when O-GlcNAcylation is insufficient due to OGT inhibition, there are not enough acetylation sites to maintain the stability of EZH2, and phosphorylation will become the domineering modification type and drive the decomposition of EZH2.

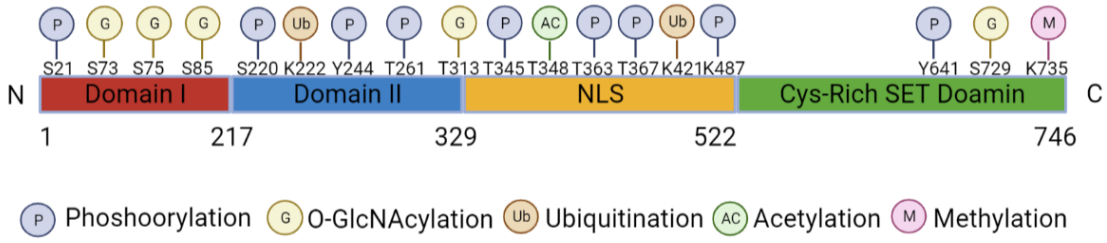

Figure 12. Overview of EZH2 PTMs. The major sites for EZH2 modifications (phosphorylation, O-GlcNAcylation, acetylation, methylation, ubiquitination) are plotted. Different colors are used to differentiate distinct modification types. Representative functions of these modifications are indicated.

O-GlcNAcylation is involved in many aspects of cellular physiology and pathophysiology, making it a potential target for the treatment of multiple diseases [80]. To date, three OGA inhibitors are currently in clinical trials for neurodegenerative diseases: MK-8719 from Merck/Alectos, ASN-120,290 from Asceneuron S.A., and LY-3,372,689 from Eli Lilly. However, OGT inhibitors still have a long way to go due to the instability and target drug delivery. Previous studies have shown that besides controlling protein stability and degradation, O-GlcNAc also regulates cellular function in multiple ways, inducing regulating conformational changes of protein folding, competing with phosphorylation, altering protein-protein interactions, modifying histones, etc., Thus, the role of O-GlcNAc in different diseases or cell types requires more comprehensive investigation in the future. In addition, we confirmed the cytotoxicity of the OGT inhibitor, OSMI-1,

which has been reported in previous studies [121]. Cellular toxicity from different OGT inhibitors has been widely reported. For example, the uracil analogue, Alloxan, first-generation OGT inhibitor that has cellular toxicity and off-target effects [122]. Ac4-5S-GlcNAc, is a compound that can lower the global level of O-GlcNAc by inhibiting OGT; however, it can also lead to the inhibition of other glycosyltransferases involved in other glycan processing events [121]. Therefore, better OGT inhibitors with low toxicity in cells are needed. The potential alternative strategy is utilizing structure-based high-throughput screening to select and evaluate different properties of molecule candidates, such as their absorption, distribution, metabolism, excretion, and toxicity [121]. In addition, although our study showed that the OGT inhibitor OSMI upregulates anti-fibrotic gene expression, we also observed different responses or sensitivities in primary cells from different IPF patients, demonstrating differences in responses to drugs between individuals which deserves further study for personalized medicine. In summary, this thesis indicated the potential mechanism of epigenetic regulation of O-GlcNAc in IPF, which also provided new evidence for future targeted drug therapy.

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