Epigenetics of Mucus Hypersecretion in Chronic Respiratory Diseases

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Abstract

Asthma, chronic obstructive pulmonary disease, and cystic fibrosis are three chronic pulmonary diseases that affect an estimated 420 million individuals across the globe. A key factor contributing to each of these conditions is mucus hypersecretion. Although management of these diseases is vastly studied, researchers have only begun to scratch the surface of the mechanisms contributing to mucus hypersecretion. Epigenetic regulation of mucus hypersecretion, other than microRNA post-translational modification, is even more scarcely researched. Detailed study of epigenetic mechanisms, such as DNA methylation and histone modification, could not only help to better the understanding of these respiratory conditions but also reveal new treatments for them. Because mucus hypersecretion is such a complex event, there are

innumerable genes involved in the process, which are beyond the scope of a single review. Therefore, the purpose of this review is to narrow the focus and summarize specific epigenetic research that has been conducted on a few aspects of mucus hypersecretion in asthma, chronic obstructive pulmonary disease, cystic fibrosis, and some cancers. Specifically, this review emphasizes the contribution of DNA methylation and histone modification of particular genes involved in mucus hypersecretion to identify possible targets for the development of future therapies for these conditions. Elucidating the role of epigenetics in these respiratory diseases may provide a breath of fresh air to millions of affected individuals around the world.

Keywords: DNA methylation; histone acetylation; epigenetics; mucus hypersecretion; respiratory diseases

Asthma is defined as a chronic inflammatory airway disorder that affects as many as 22 million individuals in the United States. It is characterized by airway hyperresponsiveness leading to transient airflow obstruction. However, some patients experience functional alterations in airway structure, including hypertrophy of smooth muscle, fibrosis, and mucus hypersecretion (1–4). Chronic obstructive pulmonary disease (COPD) is differentiated from asthma by sustained airflow limitation, most commonly induced by chronic exposure to cigarette smoke (CS), and is also defined by the aforementioned structural airway alterations. Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Among a variety of

functions, *CFTR* assists in regulation of mucus and clearance, acting as a phosphorylation-regulated anion channel (1, 2).

Mucus, comprised of mucin glycoproteins, is an innate bulwark against toxic chemicals and pathogens, acting through entrapment and mucociliary clearance (1, 2, 4, 5). Mucus secretion in human lung tissue is a highly regulated process. Goblet cells on surface epithelium and mucous cells in submucosal glands are responsible for the production and secretion of mucins. More specifically, goblet cells possess secretory granules that store and secrete mucins, which are then released via exocytosis. They then fuse to their target cell membrane to release the mucins. Baseline numbers of goblet cells

can vary throughout different parts of the human airway, but they are sufficient to produce an innate immunity. Comparatively, in patients with asthma, patients with COPD, and patients with CF, goblet cells are found in much greater abundance in both normal and abnormal areas, which can lead to elevated production of mucins and therefore mucus hypersecretion (1, 2, 4). Although the exact role of mucus hypersecretion in airway hyperreactivity has not been fully elucidated, it can cause significant mucus plugging, which is well documented as a principal cause of death in patients with asthma (6).

The mucin (*MUC*) genes discussed in this review are *MUC5AC* and *MUC5B*. *MUC5AC* and *MUC5B* have been found to

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have the most substantial involvement in asthma, COPD, and CF. Notably, recent data reported by Davis and Dickey suggest that, in healthy subjects, MUC5B is the predominant mucin produced and that the amount produced does not accumulate pathologically in airways (7). However, in a recent study, Helling and colleagues examined the role of *MUC5B* in interstitial pulmonary fibrosis (IPF); thus, it will be discussed in this review (8). Factors implicated in the synthesis of mucin and mucus hypersecretion in respiratory diseases have been thoroughly studied. The factors of *MUC5AC* expression most relevant to this review are CS, LPS, and pyocyanin, which act through Toll-like receptor-4 (TLR-4) and TLR-2 or TLR-6, respectively; epidermal growth factor (EGF) and its receptor (EGFR); IL-13; signal transducer and activator of transcription 6 (STAT6); and TNF- α (9–14). Chronic CS exposure can lead to a prolonged inflammatory response that is essentially constitutive upregulation of acute-phase reactants and stimulation of the adaptive immune response, eventually inducing release of T-helper cell type 2 (Th2) cytokines such as IL-13 and upregulation of *MUC5AC* production (5, 15–17). CS also stimulates activation of the EGFR pathway, which acts through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, specifically STAT1 and STAT3, to induce production of the transcription factor hypoxia-inducible factor-1 (HIF-1), which subsequently increases *MUC5AC* production (5). Bacterial products such as LPS and pyocyanin are recognized by their respective TLRs as part of the innate immune response. These TLRs act through secondary messenger pathways to activate the transcription factors NF- κ B and activator protein-1 (AP-1), both of which have been reported to upregulate *MUC5AC* production (5) and are further discussed in this review. Kuperman and colleagues demonstrated, in two separate studies, that *STAT6*-knockout mice experienced no increase in airway goblet cell formation after exposure to ovalbumin (OVA), which is normally mediated by IL-13 (14). Mice with intact *STAT6* and overexpression of IL-13 experienced mucus hypersecretion, whereas *STAT6*-knockout mice with IL-13 overexpression did not. Reconstitution of *STAT6* in the knockout mice with IL-13 overexpression developed

airway mucus hypersecretion, but to the same degree as seen in the wild-type mice (14). However, *MUC5AC* has no STAT6 binding sites; thus, an additional factor proposed to play a role in this pathway includes forkhead box A2 (FOXA2) (18, 19). Wan and colleagues found that in both human and animal models, downregulation of FOXA2 expression led to goblet cell hyperplasia (19). Lappalainen and colleagues found that downregulation of FOXA2 increased the expression of *MUC5AC*-positive goblet cells in mice overexpressing IL-1 β (20). Zhen and colleagues reported that FOXA2 downregulation is mediated by both the IL-13 and EGFR pathways, which correlates with an increase in *MUC5AC* transcription (21). HIF-1 is believed to mediate IL-13-induced upregulation of *MUC5AC* (5). The mechanisms regulating *MUC5B* expression are not as well studied, but IL-17, IL-6, and the STAT6-STAT3 pathway have been implicated (Figure 1) (9, 22).

Epigenetics is a growing field that displays great promise for the regulation of mucus hypersecretion. Epigenetics can be defined as a series of changes in gene expression that do not alter the underlying sequence of DNA (23, 24). The epigenetic mechanisms contributing to mucus hypersecretion discussed in this review are DNA methylation and histone modification. DNA methylation is defined as the addition of a methyl group to cytosine residues in CpG islands in the promoter region of a gene. Methylation occurs via DNA methyltransferases (DNMTs) and usually silences genes. Acetylation or deacetylation via histone acetyltransferases (HATs) or HDACs, respectively, is one form of post-translational modification that can lead to increased or decreased transcription of genes, respectively (23, 25). HATs induce acetylation, whereas HDACs are responsible for deacetylation (23, 26). HDAC inhibitors (HDACIs) can be used to prevent expansion of tumor cells by arresting the cell cycle and stimulating apoptosis. Hyperacetylating histones can induce either expression or repression of genes (23, 27). HDACIs may indirectly silence genes by inducing hyperacetylation-dependent activation of genes that act similarly to tumor suppressor genes, which then inactivate other genes (23).

Publications on epigenetics in relation to mucus hypersecretion, aside from studies focused on microRNAs (28–30), are still scarce. However, there is ample research on the molecular mechanisms contributing to asthma, COPD, and CF, which may reveal connections between epigenetic regulation and the mechanisms of mucus hypersecretion. This review focuses purely on DNA methylation and histone modification of genes.

MUC Gene Regulation

The role of epigenetic regulation of *MUC5AC* and *MUC5B* has been thoroughly researched in numerous cancers, and the concepts elucidated could possibly be applied to mucus hypersecretion in chronic pulmonary diseases (31–34). These mucins are transcribed from genes on chromosome 11p15 and expressed on normal lung tissue. Furthermore, their overexpression is correlated with development of chronic respiratory conditions and pancreatobiliary carcinomas (35–40).

Yamada and colleagues discovered the epigenetic regulation contributing to this phenomenon by quantifying the methylation status of *MUC5AC* in human lung, breast, colon, and pancreatic cancer cell lines (41). Certain cell lines were found to have *MUC5AC* overexpression, whereas others did not. Yamada and colleagues then treated low *MUC5AC* expression cell lines with 5-aza-2'-deoxycytidine (5-AzaC), a DNA-demethylating agent, to determine whether DNA methylation played a role in regulating *MUC5AC* expression. These cell lines underwent a drastic increase in *MUC5AC* expression after 5-AzaC led to hypomethylation of the CpG islands near the promoter region of the *MUC5AC* gene. Renaud and colleagues demonstrated similar findings in colon cancer (42). Thus, hypomethylation of *MUC5AC*, with subsequent overexpression of *MUC5AC*, may also contribute to mucus hypersecretion in pulmonary diseases. Vincent and colleagues presented conflicting data, however, when they found that DNA methylation did not have a significant effect on *MUC5AC* expression in pancreatic, gastric, and esophageal adenocarcinoma cell lines (43). They proposed that this may be due to the fact that the *MUC5AC* promoter has fewer CpG

Figure 1. Major pathways involved in mucus hypersecretion. Cigarette smoke is believed to constitutively upregulate acute-phase reactants and stimulate the adaptive immune response that leads to production of IL-13. Allergic inflammation also stimulates the adaptive immune response to increase production of IL-13. IL-13 upregulation of MUC5AC production is mediated by hypoxia-inducible factor-1 (HIF-1). Cigarette smoke also activates the epidermal growth factor receptor (EGFR) pathway, which then acts through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway to induce HIF-1-mediated upregulation of MUC5AC. Downregulation of forkhead box A2 (FOXA2) and hypersecretion of IL-13 contribute to STAT6-mediated production of HIF-1, which in turn stimulates MUC5AC production. LPS and pyocyanin (PCN) act through their respective Toll-like receptors (TLRs) to induce production of NF- κ B and activator protein-1 (AP-1), which upregulate MUC5AC. Although the processes regulating MUC5B production are not well understood, IL-6, IL-17, and STAT6-STAT3 are believed to be involved.

dinucleotides, as well as to the possibility that other mechanisms may contribute to *MUC5AC* silencing by hypermethylation. Ho and colleagues reported similar results (44). However, Vincent and colleagues discovered that demethylation of *MUC2* and *MUC5B* promoter regions with 5-AzaC led to significant upregulation of their expression (43). Yamada and colleagues' data reflected this as well regarding *MUC2* (41, 45, 46). In the context of pulmonary disease, Brand and colleagues found that animal airways with goblet cell hyperplasia resulting from sensitization with OVA experienced an overall decrease in airway inflammation and hyperreactivity after treatment with 5-azacytidine (5-Aza) (47). Although they did not comment on the specific effect of 5-Aza on goblet cell hyperplasia and mucus secretion in their study, it can be inferred from the work of Vincent and colleagues and Yamada and colleagues that 5-Aza may have had some effect on *MUC5AC* and *MUC5B* expression via demethylation (41, 43, 47). Taken together, hypermethylation and silencing

of *MUC5AC* and *MUC5B* should be examined as a therapeutic mechanism for treating asthma, COPD, and CF (48).

Song and colleagues examined epigenetic regulation of *MUC* gene expression via silencing of the transcription factor sterile α -motif–pointed domain-containing Ets-like factor (SPDEF) in lung epithelial cells, which normally stimulates *MUC5AC* expression (49). They found that fusing zinc finger proteins to DNMT3A and G9A induced targeted hypermethylation silencing of *SPDEF*, which induced decreased production of *MUC5AC* in mouse lung epithelial cells. It would be interesting to see if these results could be replicated in human lung tissue. *MUC* gene regulation is discussed further, but in the context of indirect epigenetic regulation.

STAT Gene Regulation

STAT3 has many roles in malignancies because of its role in the formation of oncogenic and inflammatory factors (50, 51). Hypomethylation or hypermethylation of

STAT3 itself, of certain genes by STAT3, or of other genes with which STAT3 interacts, has been shown to contribute to mucus hypersecretion in some STAT3-associated oncogenic pathways (50, 51). Guang and colleagues postulated that *Helicobacter pylori* increases mucin expression by demethylation of CpG islands near the *MUC1* promoter region, leading to increased binding of STAT3 to *MUC1*, which amplifies *MUC1* expression and mucin production (52). Supporting this hypothesis, some studies have suggested that the link between *Helicobacter pylori* and gastric cancer is a result of epigenetic changes, including DNA methylation near CpG islands in promoter regions (53–55). Thus, demethylation of either *MUC5AC* or *MUC5B* could allow for increased STAT3 binding, further perpetuating activation of the STAT6-STAT3 pathway and leading to mucus hypersecretion. In fact, Hao and colleagues demonstrated that the STAT6-STAT3 pathway is heavily involved in *MUC5AC* and *MUC5B* expression (22).

Mycoplasma pneumoniae is known to cause mucus hypersecretion in bronchial

airways (56–59). Hao and colleagues found that *M. pneumoniae* led to activation of both the STAT6-STAT3 signaling pathway and EGFR signaling pathways (22). This led to increased *MUC* expression, specifically *MUC5AC* and *MUC5B*. Activation of both pathways further exacerbated mucin synthesis by downregulating FOXA2 (Figure 2), which normally suppresses transcription of mucins. Although Hao and colleagues did not examine epigenetic regulation, possible epigenetic defects contributing to mucus hypersecretion can be theorized.

Al-Jamal and colleagues conducted an *in vitro* study examining demethylation of Src homology 2-containing protein tyrosine phosphatase 1 (*SHP-1*) in the context of STAT3 regulation and the response of patients with acute myeloid leukemia (AML) to chemotherapy (60). *SHP-1*, a tyrosine phosphate tumor suppressor gene, normally negatively regulates the JAK/STAT pathway. Studies have shown that suppression of *SHP-1*

through DNA methylation leads to upregulation of STAT3 and subsequent development of AML. Al-Jamal and colleagues hypothesized that this *SHP-1* hypermethylation-induced STAT3 upregulation may play a role in resistance to certain chemotherapeutic agents used in subjects with AML. Al-Jamal and colleagues further surmised that reversing the methylation of *SHP-1* in patients with *FLT3* internal tandem duplication (*FLT3-ITD*) AML may lead to downregulation of STAT3 in the AML pathogenic process. To test this theory, they treated *FLT3-ITD* cells with 5-Aza, a chemotherapeutic drug that inhibits DNMT enzymes, thus leading to hypomethylation of DNA. They then compared the degrees of methylation of the *SHP-1* gene and the expression of *SHP-1* protein before and after the addition of 5-Aza. As expected, hypomethylation of the *SHP-1* promoter region in the 5-Aza-treated *FLT3-ITD* AML cells led to inhibition of STAT3 expression (60). Jang

and colleagues found that *MUC5AC* airway expression was significantly reduced in *SHP-1*-deficient mice exposed to oxidative stress when compared with *SHP-1*-positive mice. This study was the first to establish a link between *SHP-1* and mucus hypersecretion in respiratory diseases (61).

Taking these mechanisms into account, it can be postulated that overactivation of STAT3 by hypermethylation and subsequent suppression of *SHP-1* may lead to increased *MUC* gene expression in respiratory conditions. 5-Aza could be used in these patients to reactivate *SHP-1* via hypomethylation of its promoter region. However, it is important to note that the demethylation triggered by 5-Aza is widespread and thus would demethylate several parts of the genome. Other methods to specifically target the *SHP-1* promoter region for demethylation need to be explored, such as coupling of CRISPR and dCas9 linked to a DNA methylase (62). CRISPR/Cas9 will be discussed further in the FUTURE DIRECTIONS section of this

Figure 2. STAT6 and STAT3 pathways in mucus hypersecretion. *Mycoplasma pneumoniae* acts as an activator of three pathways: STAT6 and STAT3 and the EGFR pathway. The STAT6 pathway induces mucus hypersecretion by binding to histone 3 (H3), then participating in the human calcium-activated protein chloride channel (hCLCA-1), followed by transcription of the *MUC5AC* gene. Hypermethylation (Me) of H3 may lead to inhibition of the STAT6 pathway. The EGFR and STAT3 pathways involve downregulation of FOXA2, which increases transcription of *MUC5B*. Treatment with IL-4 or IL-13 was found to deplete FOXA2 in the EGFR and STAT3 pathways, whereas *Pneumocystis* was shown to elevate concentrations of hCLCA-1 in the STAT6 pathway. P = phosphate.

review. SHP-1 has also been shown to regulate reactive oxygen species (ROS) concentrations, which contribute to airway mucus hypersecretion triggered by oxidative stress. Yu and colleagues also implicated ROS in STAT3 activation-induced angiogenesis in glioblastoma pathogenesis (63). Their study also showed that the antioxidant *N*-acetylcysteine led to downregulation of STAT3 in glioblastoma cells. Thus, it can be theorized that ROS-induced activation of STAT3 in patients with COPD who experience chronic hypoxia and subsequent airway ROS production could also contribute to airway mucus hypersecretion. Based on these data, 5-Aza's effectiveness could theoretically be augmented by antioxidants such as *N*-acetylcysteine, either parenterally or inhalationally.

Li and colleagues examined the role of folic acid in the alteration of DNA methylation in Alzheimer's disease (64). They found that mouse Neuro-2a cells expressing human amyloid precursor protein 695 treated with folic acid had more heavily methylated DNA than those not treated with folic acid. Specifically, they discovered that certain genes within the JAK/STAT pathway, including *STAT3*, became hypermethylated and subsequently silenced. This led to hypoattenuated expression of IL-13 and other stress markers, which contribute to Alzheimer's disease. As discussed previously, IL-13 is also well known to induce airway mucus hypersecretion by stimulating MUC5AC production (65, 66). Based on these results, folic acid supplementation could be used as a therapeutic modality to directly methylate *STAT3* and attenuate airway mucus secretion by decreasing *STAT3* expression and *IL-13* expression. Interestingly, in a case-control study, Hirayama and colleagues examined folic acid intake in Japanese patients with COPD and showed that inadequate folic acid intake not only correlated with worsening of COPD symptoms, but also increased the risk of developing COPD (67). Adequate or high folic acid intake, however, correlated with improved spirometry results, decreased symptom severity, and decreased prevalence of COPD. Although Hirayama and colleagues did not examine the role of epigenetics in this phenomenon, it provides an excellent foundation for examining whether folic acid-induced

methylation of *STAT3* would produce similar results. Additional studies could be performed to examine if coadministration of tiotropium, which has been shown by Kistemaker and colleagues to downregulate IL-13-induced goblet cell metaplasia in human airways (68), may increase the efficacy of folic acid. Concomitant use of IL-13 antibodies and folic acid may also enhance symptomatic relief. A number of review articles have documented conflicting data on the role of folic acid in the development of asthma. High-dose prenatal folic acid supplementation in the first trimester has been associated with increased childhood wheezing (69), and several studies have linked high folic acid intake in childhood to increased wheezing and atopic disease (70). These studies had a number of limitations, however, and in a recent large meta-analysis, Wang and colleagues did not find any correlation with development of asthma or increased wheezing in children (71). Thus, it may be beneficial to examine whether folic acid decreases mucus production in patients with asthma.

Another negative regulator of *STAT3* expression, suppressor of cytokine signaling 3 (SOCS-3), has been shown to contribute to COPD and asthma pathogenesis, but it appears to have conflicting roles. Springer and colleagues revealed that patients with COPD exhibited decreased expression of SOCS-3, presumably secondary to CS exposure (72). Nasreen and colleagues found that this downregulation of SOCS-3 could be reversed with a combination of fluticasone propionate and salmeterol, as well as with fluticasone furoate (73, 74). However, Gao and colleagues found that increased concentrations of SOCS-3 in the IgG immune complex rodent lung model led to an increase in lung inflammation by IL-6 (75–77). This likely was due to the fact that IL-6 activates *STAT3*. Thus, although SOCS-3 normally directly inhibits *STAT3*, its overexpression can indirectly activate *STAT3* by increasing IL-6 concentrations. Similar studies reflect these conflicting results (78). However, several studies of other inflammatory conditions have shown that hypomethylation and subsequent silencing of *SOCS-3* significantly contributes to their pathogenesis. Gupta and colleagues and Dhar and colleagues found that coadministration of TNF- α and insulin-like growth factor 1 led to upregulation of DNMT1 and subsequent

hypermethylation and silencing of *SOCS-3* in the coronary smooth muscle cells of pigs (79, 80). In their review of epigenetic regulation of *SOCS-3*, Boosani and colleagues theorized that these results contribute to atherosclerosis by preventing *SOCS-3*-mediated *STAT3* inhibition, as well as by IL-6-induced activation of *STAT3* and silencing of *SOCS-3* (30). Li and colleagues further supported this in the context of ulcerative colitis-associated colorectal cancer when they discovered that colonic cells from patients with ulcerative colitis possessed highly methylated *SOCS-3*, as well as impaired upregulation of *SOCS-3* in the presence of IL-6 (81). They also found that IL-6 stimulated DNMT1, which then methylated and silenced *SOCS-3*. This hypermethylation of *SOCS-3* was reversed with 5-AzaC and led to inhibition of IL-6-induced *STAT3* activation. Examining these studies collectively, one could propose that inhibition of DNMT1 directly or removal of methyl groups from *SOCS-3* via 5-AzaC could be used alone or in combination with fluticasone propionate/salmeterol or fluticasone furoate to reverse *SOCS-3* silencing and inhibit activation of *STAT3*. Predictably, this would in turn decrease mucus hypersecretion in human airways. However, given the conflicting data regarding elevated *SOCS-3* concentrations in airway diseases as described above, these therapies may require supplementation with IL-6 inhibitors, such as tocilizumab and siltuximab, to reach their full effectiveness (82).

Maximal *STAT6* binding occurs between areas of histone 3 that are trimethylated (i.e., between histone 3 lysine 4 trimethylation areas on histone 3) (83–86). It is known that elevated concentrations of chloride channel accessory 1 (hCLCA1), a calcium-sensitive chloride conductance protein, plays a large role in mucus hypersecretion in patients with COPD and rats colonized with *Pneumocystis*-related organisms (1, 87–89). This occurs via hCLCA1-induced overproduction of MUC5AC (89, 90). Pérez and colleagues recently revealed that immunocompetent infants with a high *Pneumocystis* burden had elevated concentrations of hCLCA1 in their lung parenchyma as well as overproduction of MUC5AC (84). Interestingly, the hCLCA1-MUC5AC pathway was found to be regulated by *STAT6*. Thus, increasing

methylation of histone 3, which is known to suppress STAT6 binding, may serve as a method of downregulating STAT6-dependent pathways, including the *Pneumocystis*-induced hCLCA1-MUC5AC pathway, thereby dampening MUC5AC production and consequent mucus hypersecretion in infants with high *Pneumocystis* colonization and in patients with COPD.

HDAC Regulation

One proposed mechanism of COPD pathogenesis is autophagic destruction of epithelial cells and ciliary shortening caused by CS (91–94). These events impair mucociliary clearance and prevent replacement of damaged epithelial cells by goblet cells (95, 96). Many signaling molecules have been implicated in ciliary shortening, including HDAC6. HDAC6 has many roles in facilitating the autophagic process, including delivery of ubiquitinated proteins to aggresomes, augmenting the fusion of autophagosomes and lysosomes, and altering microtubule function through destabilization and deacetylation (97–101). To better elucidate this correlation, Lam and colleagues examined ciliary length in tracheal epithelial cells of mice exposed to CS and found a dose-dependent increase in the destruction of epithelial cells, a decrease in ciliary length, and an increase in production of autophagosomes (102). These results recapitulated the characteristics of human epithelial cells exposed to CS extract. Lam and colleagues also found that upregulated expression of HDAC6 in mouse tracheal epithelial cells was triggered by CS exposure. To determine a correlation, they exposed mice deficient in HDAC6 (HDAC^{-/-}) to CS, and they found that autophagic activity was decreased in the tracheal epithelial cells owing to decreased fusion of autophagosomes and lysosomes. This suggests that HDAC6 (HDAC^{+/+}) suppression protects cilia from shortening and inhibits destruction of epithelial cells. Lam and colleagues then examined the methylation status of HDAC6 in epithelial cells from patients with and without COPD who smoked and in nonsmokers. They found that HDAC6 was more hypomethylated in smokers with and without COPD, thus leading to increased expression of HDAC6 (102). Thus,

methylation of HDAC6 or use of a targeted HDACI could represent a possible therapeutic modality.

Grausenburger and colleagues used *Cd4-Cre*-knockout mice to create T cells devoid of HDAC1 (HDAC1^Δ mice) and compared the inflammation generated with that of T cells with intact HDAC (HDAC1^F) (103). After being challenged with the allergic antigen OVA, HDAC1^Δ murine T cells produced a more robust Th2 response than HDAC1^F mice, eventually leading to development of asthma-like characteristics such as BAL fluid eosinophilia, lung inflammation, and mucus hypersecretion. This was supported by evidence that HDAC1^Δ mice T cells shifted more toward producing cytokines responsible for a Th2 response than controls. HDAC1^Δ murine T-helper cell type 1 (Th1) cells released higher concentrations of IFN- γ and fewer Th1-related cytokines. HDAC1^Δ murine Th2 cells produced Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13, at much higher concentrations than HDAC1^F mice did. Thus, it was concluded that HDAC1 is critical for influencing the extent of an allergic airway response, including mucus secretion, as well as regulating the balance between the Th1 and Th2 responses. Upregulation of HDAC1 in the T cells of these mice may lead to attenuation of the allergic airway response and thus dampen or prevent mucus hypersecretion (Figure 3) (103).

Nie and colleagues examined mucus hypersecretion in three different rat COPD models, which included COPD induced by CS alone, COPD induced by CS and pretreatment with LPS, and COPD induced by CS and post-treatment with LPS (104). As described previously, LPS induces MUC5AC overproduction by activating TLR4, which activates IL-1R, I κ B, and MAPK pathways (91, 105, 106). These pathways are responsible for regulating transcription factors such as NF- κ B and AP-1, which are involved in producing MUC5AC (107, 108). HDAC2 is known to repress NF- κ B and AP-1; thus, it may contribute to the inhibition of MUC5AC overproduction. Its expression has also been shown to be decreased in rats exposed to CS and in the lung parenchyma of patients with COPD (109–111). Nie and colleagues found that all three of these groups of rats experienced mucus hypersecretion in their lung parenchyma and that transcription factors involved in

Figure 3. T-helper cell type 2 (Th2) response in mucus hypersecretion. Inhibition of HDAC1 can lead to mucus hypersecretion through a Th2-mediated response. Antigen (Ag) stimulation serves as an activator of T cells. Upregulation of HDAC1 may inhibit this pathway.

MUC5AC overproduction, such as TLR4, EGFR, AP-1, NF- κ B, and MAPKs, were upregulated (104). Also, HDAC2 was found to be downregulated, which likely played a role in MUC5AC overproduction (112). Thus, either targeted upregulation of HDAC2 expression or targeted inhibition of TLR4 or any of its downstream transcription factors, such as NF- κ B or AP-1, could be potential therapeutic agents in COPD, asthma, or CF. In fact, in a recent study, Liu and colleagues found that 7,4'-dihydroxyflavone (7,4'-DHF) may act as an HDAC2 upregulator, leading to decreased mucus hypersecretion in murine airways (Figure 4) (113). They treated human pulmonary epithelial cells with PMA, which increased MUC5AC transcription fourfold. However, 7,4'-DHF reversed this increase by preventing activation of NF- κ B, phosphorylation of STAT6, and upregulation of HDAC2. In accordance with previous studies already referenced (112), upregulation of HDAC2 via treatment with 7,4'-DHF suppressed MUC5AC transcription (113).

As discussed above, Vincent and colleagues found that demethylation of MUC2 and MUC5B with 5-AzaC led to increased expression of these genes in various adenocarcinoma cells. They also found that HDAC2 inhibited DNMT1, which prevented silencing of MUC2 and MUC5B. Addition of trichostatin A (TSA), an HDACI, led to inhibition of HDAC2, enhanced DNMT1 activity, and decreased expression of MUC2 and MUC5B. These results contradict the aforementioned data (43). Thus, though HDACI versus HDAC2

Figure 4. MUC5AC upregulation pathways. Hypomethylation of the gene *MUC5AC* and stimulation of the AP-1 and NF- κ B pathways both upregulate MUC5AC. Downregulation of HDAC2 stimulates the NF- κ B and AP-1 pathways, but it can be reversed by molecules such as 7,4'-dihydroxyflavone (7,4'-DHF). Similarly, hypomethylation may be counteracted by hypermethylation to prevent upregulation of MUC5AC and mucus hypersecretion.

may help decrease airway mucus hypersecretion via *MUC2* and *MUC5B* suppression, it may in turn lead to upregulation of *MUC5AC*. Royce and colleagues also conducted a study in which BALB/c mice with allergic airway disease were given TSA (114). They found that the TSA mice had a statistically significant decrease in the number of airway goblet cells. Similarly, upregulation of HDAC2 may decrease MUC5AC-induced mucus hypersecretion but then lead to an increase in *MUC2* and *MUC5B* expression. HDACIs and HDAC2 enhancers would have to be highly specialized to be truly efficacious, which would likely be difficult to achieve.

FOX Regulation

As discussed earlier, FOXA2 normally acts to suppress the expression of mucins. Multiple studies definitively depict FOXA2 dysregulation contributing to mucus hypersecretion. Wan and colleagues noted that selective mutation of FOXA2 in mice before lung formation is fatal; thus, it must contribute to lung morphogenesis and homeostasis both pre- and postnatally (19). They targeted FOXA2 in the respiratory epithelial cells of developing murine lungs for deletion and found that this led to goblet cell hyperplasia and an increase in mucin production, airspace enlargement, and neutrophilic infiltration. Supporting this, they found decreased or absent expression of FOXA2 in airway epithelial cells treated with IL-4, IL-13, and OVA sensitization, which led to deletion of the gene encoding surfactant protein C (*SP-C*) and subsequent goblet cell hyperplasia.

Park and colleagues (115) supported these findings by showing that increased expression of FOXA2 in murine airway epithelial cells decreased allergen-triggered mucus metaplasia by nearly 45%. They also found that the airway epithelial cells of patients with asthma had decreased concentrations of FOXA2 and increased MUC5AC concentrations. They proposed that further research into regulation of FOXA2 expression could lead to the development of novel therapies for patients with asthma to decrease mucus production.

Several studies have recently linked overexpression of MUC5B to the development of IPF. Seibold and colleagues (116) demonstrated that the strongest risk factor for developing either familial or sporadic IPF was a gain-of-function mutation of the *MUC5B* promoter region, rs35705950, which Helling and colleagues (8) discovered occurs via a G-to-T transversion in the 5' flanking region. Helling and colleagues were able to identify an enhancer region containing the rs35705950 variant, which binds RNA polymerase II and FOXA2. They also found that increased methylation of this area induced overexpression of MUC5B. Helling and colleagues theorized that hypermethylation of the area surrounding the FOXA2 binding site may lead to overproduction of MUC5B (8).

Future Directions

New advancements in the field of epigenetic research may play a vital role in future treatments, especially regarding research on DNA methylation. Until recently, DNA

methylation was researched solely via quantitative analysis of CpG dinucleotides by measuring concentrations of 5-methylcytosine (5 mC). However, Stewart and colleagues published their research examining the benefits of measuring 5-hydroxymethylcytosine (5 hmC) instead of 5 mC in quantifying the extent of DNA methylation (117). It has been postulated that 5 hmC may be an intermediary in DNA demethylation, and it may be a new contender in the realm of epigenetic regulation (118). Stewart and colleagues used a modified form of oxidative bisulfite (oxBS) chemistry via combination with the 450K DNA methylation microarray (oxBS-450K) to quantify not only 5 mC but also 5 hmC. This oxBS-450K chemistry not only is able to achieve results comparable with the current gold standard bisulfite conversion kits but also is easily reproducible. In addition, by creating a single workflow for detecting 5 hmC and 5 mC, as opposed to using multiple workflows with current bisulfite conversion kits, oxBS-450K allows for faster and more cost-effective DNA methylation analysis (117, 119). More recently, it has been shown that TET proteins can demethylate areas of DNA via TET dioxygenase-mediated oxidation of 5 mC to 5 hmC, 5-formylcytosine, and 5-carboxylcytosine, with subsequent base excision repair via thiamine DNA glycosylase (120). Even more exciting is genome editing, specifically epigenome editing with CRISPR/Cas9. Coupling of dCas9 to proteins can help direct enzymes and thus aid in regulation of histone modifications or modification of DNA methylation patterns (62). This could potentially solve the issue of generalized demethylation of DNA induced by 5-Aza, as previously discussed.

Conclusions

Use of epigenetic modifications to develop medical therapies is quickly becoming an appealing field of study. In theory, researchers can essentially influence a gene to behave in a controlled manner without permanently altering a subject's DNA sequence. Groundbreaking research examining the potential for epigenetic therapies in malignancies has been performed, paving the way for epigenetic therapies that could be applied to mucus hypersecretion in pulmonary diseases (121–123). However, the epigenetic

regulation behind pulmonary mucus hypersecretion in asthma, COPD, and CF appears to remain a relatively untapped resource for better understanding of the development of these diseases. Further research into these topics may provide a wealth of information for future treatment

of these conditions. Using epigenetic regulation provides more control in choosing specific targets to either upregulate or downregulate and thus allows researchers to more easily predict the outcomes of these therapies. There is still much to be gleaned from research

examining the pathogenesis of mucus hypersecretion, but epigenetics may provide a new frontier for better management and possible cure of these crippling diseases. ■

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