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Methyltransferase-like 3 (METTL3) Epigenetically Modulates Glutathione Peroxidase 4 (GPX4) Expression to Affect Asthma

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ABSTRACT

Asthma, a prevalent chronic airway inflammatory condition, poses a significant health challenge. In this study, we delved into the regulatory mechanisms governing asthma, focusing on Methyltransferase-like 3 (METTL3).

Through an ovalbumin (OVA)-induced mouse model and interleukin-13 (IL-13)-induced cell model, we mimicked the in vivo and in vitro functions of METTL3 in asthma.

Our research revealed that METTL3 expression significantly decreased in asthma-induced mice and IL-13-stimulated cells compared to the control group. Moreover, METTL3 overexpression enhanced bronchial epithelial cell viability and proliferation. Mechanistically, we observed elevated levels of total iron, Fe^{2+}, malondialdehyde (MDA), lipid reactive oxygen species (ROS), alongside reduced glutathione (GSH) levels in IL-13-stimulated cells. Remarkably, METTL3 overexpression counteracted these effects, suggesting a pivotal role in mitigating asthma-related oxidative stress. Furthermore, our study highlighted the involvement of N6-methyladenosine methylation (m6A) modification, where METTL3 regulated the m6A modification of glutathione peroxidase 4 (GPX4) RNA, impacting RNA stability. Knockdown of METTL3 suppressed m6A modification on GPX4 RNA, impairing its stability and contributing to IL-13-induced ferroptosis. Interestingly, METTL3 overexpression not only inhibited cell ferroptosis but also alleviated asthma symptoms.

Our findings shed light on the epigenetic regulation of asthma through METTL3-mediated m6A modification, offering potential therapeutic avenues for this prevalent inflammatory disease.

Keywords Asthma; Bronchial disease; Epithelial cells; Ferroptosis; Glutathione peroxidase 4; METTL3 protein, human; Oxidative stress; RNA stability

INTRODUCTION

Asthma is a common chronic inflammatory disease of the airways that causes bronchial inflammation, airway edema, airflow obstruction, airway hyperresponsiveness, and mucus secretion.[1,2] Asthma

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prevalence is now quite high in all age groups including adults and infants worldwide and it is increasing every year.[1] Asthma is a chronic inflammatory airway disease that is tightly linked to many cell types and intracellular components.[3,4] Studies have revealed that environmental variables (air pollution, virus infections, inhaled allergens) and genetic factors have been implicated in the development of asthma.[5]

Ferroptosis is a regulated oxidative cell death that generates depending on iron-induced lipid peroxidation.[6,7] During ferroptosis, the ions induce excessive reactive oxygen species (ROS) accumulation in the cells and the anti-oxidation process is attenuated due to restricted functions of glutathione peroxidase 4 (GPX4), which exacerbates lipid peroxidation and destroys lipid oxide metabolism.[8] GPX4 suppresses ferroptosis by using reduced glutathione and catalyzing the formation of lipid alcohols from phospholipid hydroperoxides.[9,10] Inhibiting GPX4 by eliminating GSH with erastin or directly targeting GPX4 with RSL3 may result in excessive lipid peroxidation and, ultimately, cell death.[11] Ferroptosis has been widely indicated in various cellular processes and diseases.[12,13] Hence, generating or reducing ferroptosis is seen as a possible therapeutic strategy for the development of clinical medicines.[14,15]

RNA modification is a valuable process that regulates gene expression and modulates cellular responses.[16] Among the documented RNA modification types, N6-methyladenosine methylation (m6A) is the most common form of mRNA alteration.[17] The m6A modification involves multiple regulators including the "writers", "readers", and "erasers".[18,19] According to a recent study, various m6A regulators, such as YTH N6-methyladenosine RNA binding protein 3 (YTHDF3) and YT521-B homology-domain-containing protein 1 (YTHDC1), are overexpressed in asthma and are associated with the immunological microenvironment and immune response.[20] Methyltransferase-like 3 (METTL3) has been identified as a catalytic component of the methyltransferase complex known as the "writer" and has been found to be dysregulated in certain malignancies.[16,21,22] However, its involvement in the pathophysiology of asthma is unknown. In this study, we used in vitro and in vivo models to assess the expression and relevance of METTL3 in asthma, and we identified GPX4 as a mediator of METTL3-regulated ferroptosis in bronchial epithelial cells.

MATERIALS AND METHODS

Animal Model

Four-week ale C57BL/6 mice were bought from Huafukang (Beijing, China) and kept in a special pathogen free (SPF) environment. The ovalbumin (OVA)-induced model was constructed to mimic asthma. In short, ovalbumin (OVA) (1 mg, Sigma, USA) was mixed with $Al(OH)_3$ gel (40 mg, Sigma, USA) in PBS (200 µL) and was intraperitoneally injected once a week for 3 weeks. After the last injection, mice were challenged with 5% OVA via intranasal inhalations for 7 days. For control, mice were given saline instead of OVA.

Cell Culture and Induction

The HBE and BEAS-2B cells were bought from Zhongshanjinqiao (China) and placed in 1640 medium (BI, Israel) that contains fetal bovine serum (10%; BI), at a 37°C incubator with a humidified atmosphere and 5% CO_2. For in vitro experiments, cells were induced by interleukin-13 (IL-13, Sigma, USA) with 10 ng/mL for 24 hours. For RNA degeneration detection, actinomycin D (5 µM, MCE, USA) was used to treat cells for 0, 3, and 6 hours, and then RNA was extracted for the following detection.

Cell Transfection

The siRNAs and overexpression vectors used in this work were synthesized by Tsingke Biotech (China). Cell transfection was performed by adopting the Lipofectamine 2000 that purchased from Invitrogen (USA) following the manufacturer's introduction.

Cell Counting Kit 8 (CCK-8)

HBE and BEAS-2B cells that received indicated treatment were digested and placed into a 96-well plate with a density of 5000 cells per well. After culture for 24 hours, CCK-8 reagent was mixed with the medium and incubated with cells for another 2 hours. Then absorbance values were detected using a spectrophotometer (Thermo, USA) at 450 nm.

Colony Formation

HBE and BEAS-2B cells were transfected with indicated siRNA and stimulated with IL-13. After digestion, cells were suspended as single cells and were inoculated into 6-well plates with 500 cells per well. The cells were cultured in a 37°C incubator for 14 days to form visible colonies, which were then dyed using

crystal violet at room temperature for 20 minutes. The colony number was counted and calculated.

Ferroptosis Hallmarks

The production of total iron and Fe^{2+} in cells was measured by an Iron assay kit (Abcam, USA). The levels of lipid reactive oxygen species (ROS), malondialdehyde (MDA), and glutathione (GSH) were determined by the BODIPY-C11 probe (Thermo, USA), MDA assay reagent (DOJINDO, Japan), and GSH assay kit (DOJINDO, Japan). All experiments were conducted according to the producer's protocols.

Quantitative Real-time PCR (qRT-PCR)

Total RNAs were collected from cells or lung tissues after homogenization in Trizol solution (Invitrogen, USA). After reverse transcription, the RNA level was quantified using the Taqman probe (Thermo, USA). Relative gene expression was quantified after normalization to internal reference β-actin following the $2^{-\Delta\Delta Ct}$ method.

Western Blotting Assay

Cells were lysed with RIPA buffer (Thermo, USA) to collect total protein. After protein concentration quantification by BCA kit (Beyotime, China), proteins were divided using the SDS-PAGE gel, followed by blotting to NC membranes. The protein blots were blocked in 5% skimmed milk and were probed in primary antibodies including anti-GPX4 (Abcam, USA) and anti-METTL13 (Abcam, USA) for one night at 4°C. The blots were then washed in PBST 3 times and were hatched with goat anti-rabbit antibody for 45 minutes. Finally, protein blots were reacted with the enhanced chemiluminescence (ECL) solution (Millipore, Germany) and captured using the imaging system.

Methylation RNA Immunoprecipitation (MeRIP) Assay

Cells were lysed to extract total RNA and the enrichment of m6A modification on mRNA was measured using a MeRIP assay kit (RiboBio, China) following the manufacturer's protocol.

Statistics

All data were exhibited as mean±SD and analyzed using the GraphPad Prism (Version 7.0) software. The difference between two experimental sets was measured by Student's *t*-test, and multiple comparisons were conducted by one-way analysis of variance (ANOVA). The comparison was regarded to be statistically significant with p value less than 0.05.

RESULTS

Expression of METTL3 in OVA-induced Murine Model and IL-13-induced Cell Model

We first established the OVA-induced murine model and IL-13-induced cell model to investigate the expression of METTL13. As shown in Figure 1A and 1B, the RNA and protein level of METTL3 was notably decreased in OVA-induced mice compared with the control mice. Similar findings were identified in the cell model, manifested by decreased expression of METTL3 in IL-13-stimulated bronchial epithelial cells (Figure 1C and 1D). The expression of GPX4 in the in vivo and in vitro model was notably suppressed under induction (Figure 1B and 1D). Moreover, overexpression of METTL3 could markedly recover the expression of METTL3 and GPX4 both in vitro and in vivo (Figure 1B and 1D).

METTL3 Promotes the Proliferation of Bronchial Epithelial Cells

We next evaluated the effects of METTL13 on the proliferation of bronchial epithelial cells. The results from CCK-8 and colony formation experiment indicated that IL-13 stimulation repressed the viability (Figure 2A) and colony number (Figure 2B) of HBE and BEAS-2B cells, whereas overexpression of METTL3 recovered cell viability and proliferation.

METTL3 Alleviates Ferroptosis of Bronchial Epithelial Cells

Subsequently, we detected the alteration of ferroptosis hallmarks in the IL-13-stimulated cell model. The levels of total iron, Fe^{2+}, lipid ROS, and MDA were significantly elevated and the level of GSH was reduced in HBE (Figure 3A) and BEAS-2B (Figure 3B) cells under IL-13 stimulation, whereas overexpression of METTL3 abolished these effects. Besides, the expression of ferroptosis inhibitor was downregulated under IL-13 induction and recovered by METTL3 overexpression (Figure 3C).

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METTL3 Epigenetically Targets GPX4 in Bronchial Epithelial Cell

To determine the molecular mechanisms underlying METTL3-regulated ferroptosis in bronchial epithelial cells, we investigated the expression of GPX4. The results from the MeRIP experiment indicated that the knockdown of METTL3 could suppress the enrichment of m6A modification on GPX4 RNA (Figure 4A), and simultaneously reduced the RNA and protein level of GPX4 in cells (Figure 4B and C). After treatment with actinomycin D for different times (0, 3, and 6 hours) to block protein synthesis, the RNA level of GPX4 was decreased faster in METTL3-depleted cells compared with the control cells (Figure 4D).

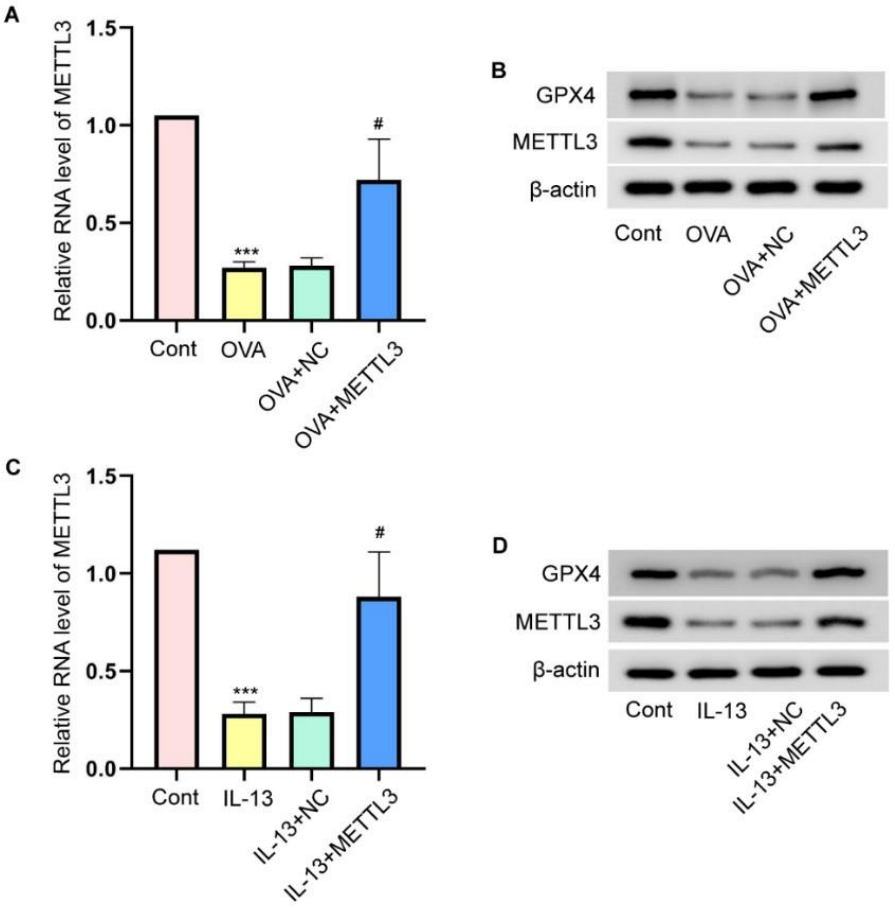

Figure 1. Expression of METTL3 in ovalbumin (OVA)-induced murine model and interleukin-13 (IL-13)-induced cell model. (A-B) The (A) RNA and (B) protein level of methyltransferase-like 3 (METTL3) in OVA-induced murine model. (C-D) The (C) RNA and (D) protein levels of METTL3 and glutathione peroxidase 4 (GPX4) in the IL-13-induced cell model.

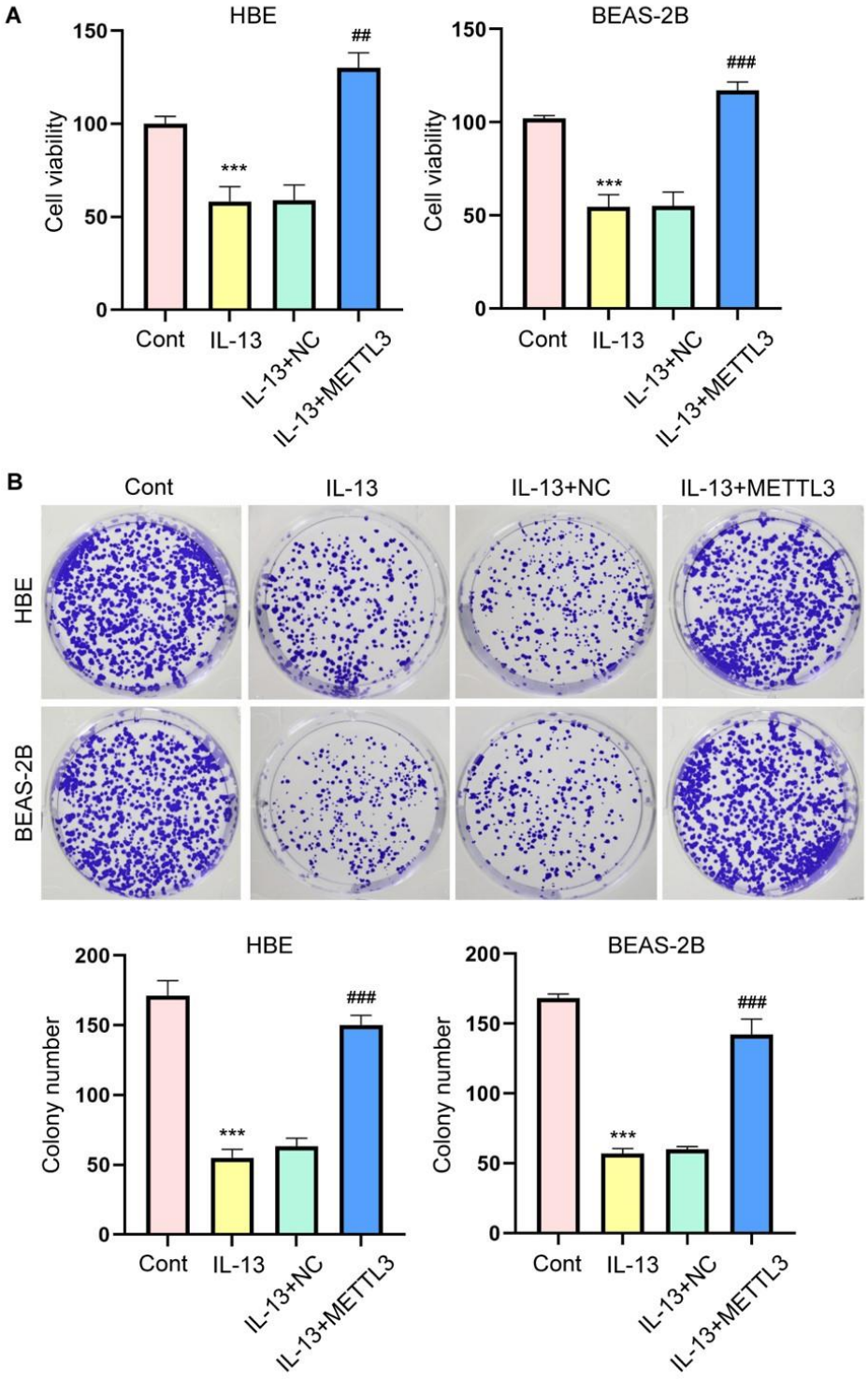

Figure 2. METTL3 promotes the proliferation of bronchial epithelial cells. (A) Cell viability of HBE and BEAS-2B cells was detected by cell counting kit 8 (CCK-8) assay. (B) Proliferation of HBE and BEAS-2B cells was measured by colony formation assay. Representative images and histograms were shown. ***p<0.001 vs control; ##p<0.01, ###p<0.001 vs IL-13 induction.

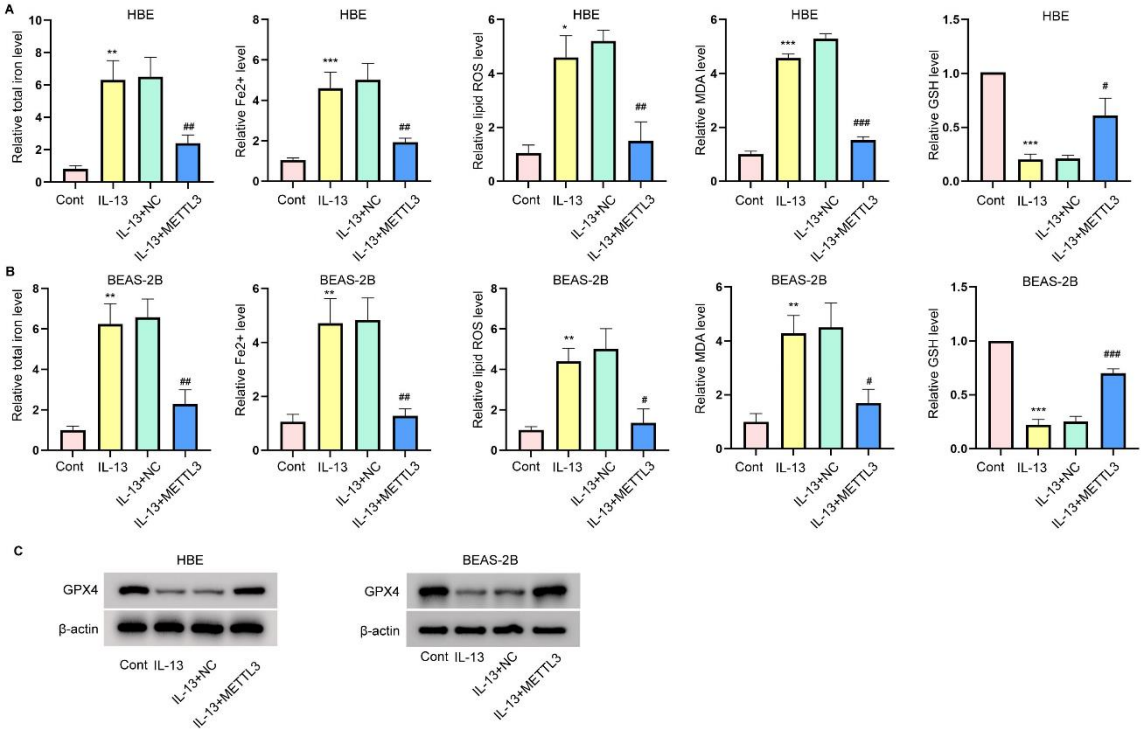

Figure 3. METTL3 alleviates ferroptosis of bronchial epithelial cells. (A) The levels of total iron, Fe^{2+}, lipid reactive oxygen species (ROS), malondialdehyde (MDA), and glutathione (GSH) in HBE cells that were stimulated with IL-13 and transfected with METTL3 overexpression vectors. (B) The levels of total iron, Fe^{2+}, lipid ROS, MDA, and GSH in BEAS-2B cells that were induced with IL-13 and transfected with METTL3 overexpression vectors. (C) Protein level of GPX4 in IL-13-induced cell model. *p<0.05, **p<0.01, ***p<0.001 vs control; #p<0.05, ##p<0.01, ###p<0.001 vs IL-13 induction.

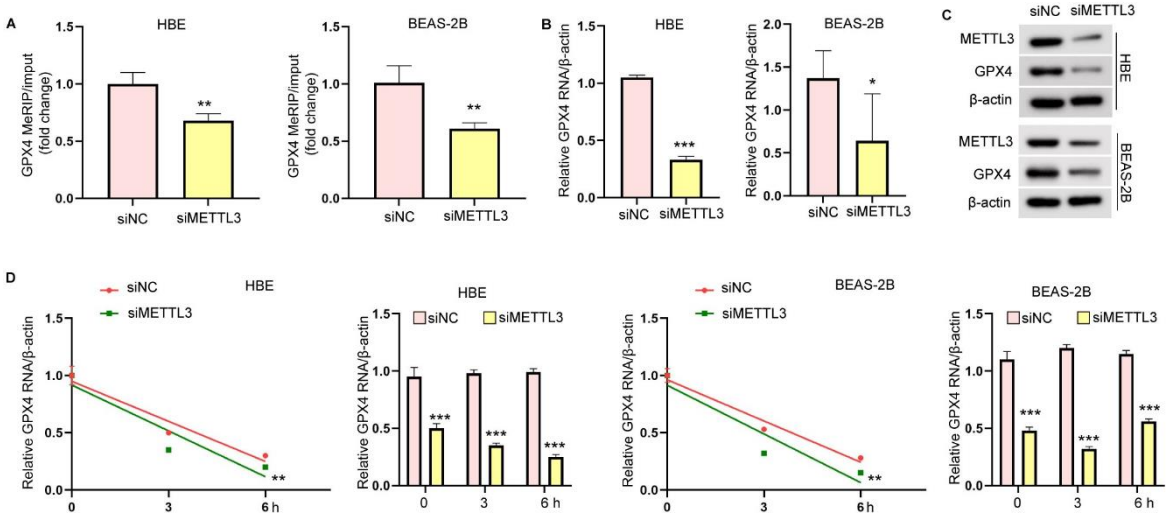

Figure 4. METTL3 epigenetically targets glutathione peroxidase 4 (GPX4) in bronchial epithelial cells. (A) The N6-methyladenosine (m6A) modification level on GPX4 RNA was measured by methylation RNA immunoprecipitation (MeRIP) assay. (B) RNA and (C) protein level of GPX4 in cells. (D) Cells were treated with actinomycin D for 0, 3, and 6hours, then the RNA level of GPX4 was measured by qCPR. *p<0.05, **p<0.01, ***p<0.001 vs siNC.

METTL3 Modulates Ferroptosis of Bronchial Epithelial Cell via GPX4

Next, we confirmed the role of GPX4 as a mediator of METTL3 in the ferroptosis of bronchial epithelial cells. We conducted overexpression of METL3 and knockdown of GPX4 in IL-13-stimulated cells and observed that overexpression of METTL3 recovered the cell viability that was repressed by IL-13 induction, whereas depletion of GPX4 abolished this effect (Figure 5A). Moreover, the IL-13 induced notable elevation of iron, Fe^{2+}, and MDA in cells was repressed by METTL13, which was then recovered by GPX4 depletion (Figure 5B and 5C). We also examined the protein expression and observed that overexpression of METTL3 recovered the GPX4 expression in IL-13-induced HBE and BEAS-2B cells, whereas knockdown of GPX4 reversed this effect without altering METTL3 expression (Figure 5D).

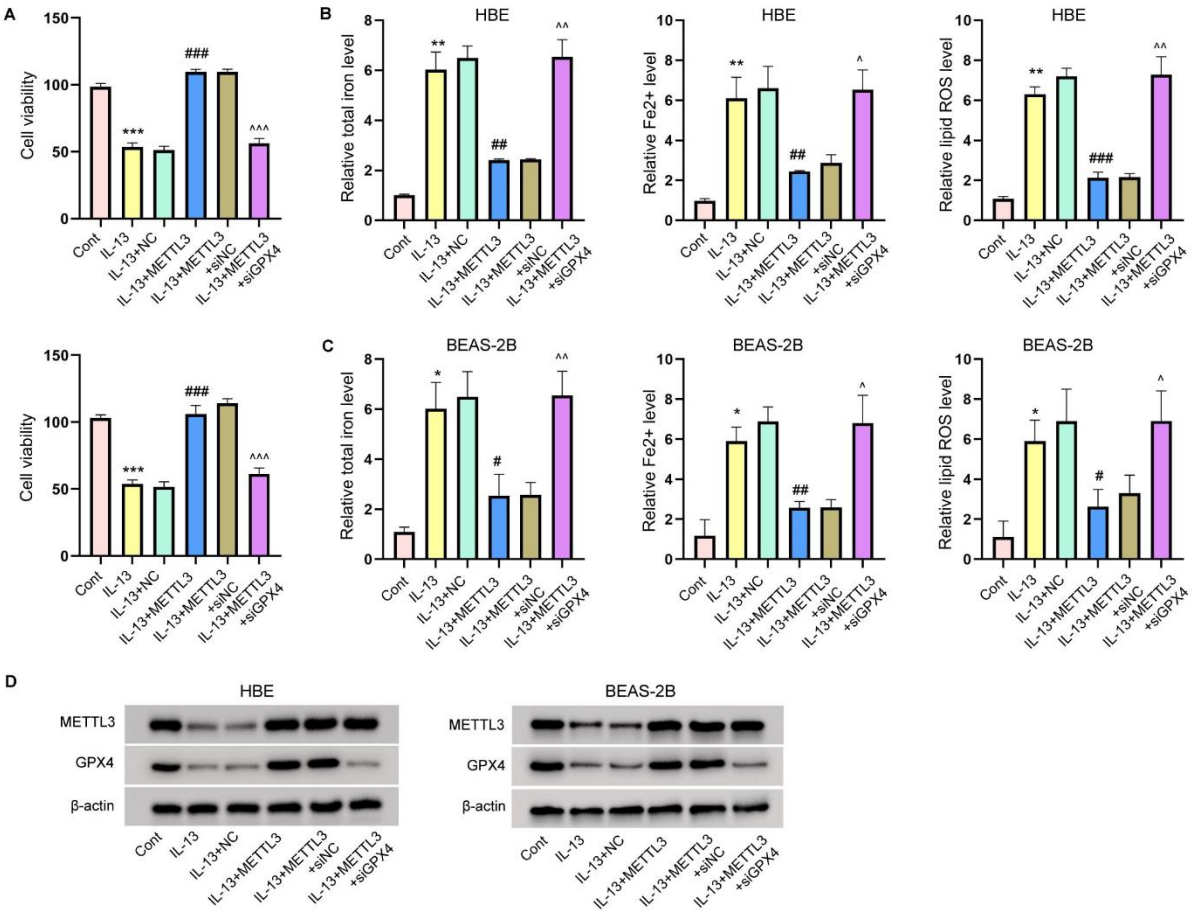

Figure 5. METTL3 modulates ferroptosis of bronchial epithelial cell via glutathione peroxidase 4 (GPX4). (A) Cell viability was measured by the Cell Counting Kit-8 (CCK-8) experiment. (B and C) The intracellular levels of iron, Fe^{2+} and 3,4-methylenedioxyamphetamine (MDA). (D) The protein level of METTL3 and GPX4 in interleukin-13 (IL-13)-induced cell model. *p<0.05, **p<0.01, ***p<0.001 vs control; #p<0.05, ##p<0.01, ###p<0.001 vs IL-13 induction; ^p<0.05, ^^p<0.01, ^^^p<0.001 vs IL-13 + GPX4 overexpression.

DISCUSSION

As a prevalent respiratory disease, asthma has become a severe social and economic burden in modern society.[23] The leukotriene receptor antagonists and glucocorticoids are the main clinical treatment reagents that are used in clinics. Nevertheless, the traditional anti-inflammation agents usually cause lots of secondary actions and threaten human health.[24] Hence, novel treatment and targeting strategies are necessary for asthma. In this work, we explored the function and potential regulatory mechanisms in METTL3-reuglated ferroptosis during asthma and identified METTL3 as a post-transcriptional regulator of ferroptosis suppressor GPX4 to repress ferroptosis of bronchial epithelial cells and alleviate asthma.

METTL3 is the catalytic subunit of the methyltransferase, which together with METTL14 forms a heterodimer to catalyze the m6A modification on mRNAs.[25] METTL3 has been reported to target various genes and exhibits both oncogenic and tumor suppressor effects on various cancers.[26] METTL3 is also involved in various inflammatory diseases, such as osteoarthritis.[27,28] METTL3 suppressed the expression of autophagy-related 7 via modulating its m6A level, which led to decreased autophagosome formation and repressed autophagy in fibroblast-like synoviocytes.[29] The METTL3-repressed autophagy exacerbated the cartilage destruction in a murine model.[29] In the renal fibrosis model, METTL3 was identified to catalyze the m6A modification and promote the maturation of miR-21-5p and the activation of a downstream nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling pathway to drive renal fibrosis.[30] Moreover, METTL3 inhibited oxygen consumption and ATP production to increase the accumulation of ROS in inflammatory monocytes, which indicated the potential pathogenesis of several inflammation-related diseases, including atherosclerosis, arthritis, and pulmonary fibrosis.[31] In this study, we identified the repressed expression of METTL3 in the OVA-induced in vivo asthma model and IL-13-induced in vitro model and found that METTL3 overexpression stimulated the proliferation of bronchial epithelial cells and suppressed cell ferroptosis.

Further study on molecular mechanisms identified GPX4 as the target of METTL3 in the asthma model. GPX4 is an important peroxidase that relieves ROS accumulation via catalyzing the GSH, consequently reducing lipid oxidation and ferroptosis in cells.[32] Accumulating studies have supported the outstanding effects of targeting GPX4 to modulate ferroptosis in the treatment of diseases.[33-35] Consistent with these studies, we identified METTL3 upregulated the expression of GPX4 to suppress ferroptosis, facilitated the viability and proliferation of bronchial epithelial cells and ameliorated asthma. Our study revealed the role of m6A modification in the pathogenesis of asthma and presented METTL3 as a potential target for asthma treatment. However, the lack of gene silence experiments limited the stringency of the current study, and the potential targets of METTL3 in asthma need further exploration.

To summarize, we identified decreased expression of METTL3 in in vitro and in vivo models of asthma, and overexpression of METTL3 increased the m6A level of GPX4 to inhibit cell ferroptosis. Our findings provided a novel mechanism for the pathogenesis of asthma involving the epigenetic regulation of ferroptosis by METTL3.

STATEMENT OF ETHICS

All experiment processes were conducted under the authorization of the Animal Ethic Committee of Second Affiliated Hospital of Fujian Medical University (Approval number: 2021001017).

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Not applicable

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