Suppression of *MUC5AC* expression in human bronchial epithelial cells by interferon-γ

Running title: Suppression of *MUC5AC* expression by interferon-γ

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Contributions

Experiments and analysis; TO, TT and AA, Conception and design; TT and HA, Significant advise; OS, HY, YN, HK and AH, Drafting a manuscript; TO and TT.

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Abstract

Background: Excessive mucin secretion in the airway is an important feature of airway inflammatory diseases. MUC5AC expression is regulated by a variety of stimuli such as cytokines. Little is known about the role of interferon (IFN)-γ in MUC5AC expression in human bronchial epithelial cells.

Methods: Human pulmonary mucoepidermoid carcinoma cell line (NCI-H292) and normal human bronchial epithelial (NHBE) cells were used to assess the effects of IFN-γ on *MUC5AC* transcription.

Results: Transforming growth factor (TGF)-α and double-stranded RNA (polyI:C)-induced MUC5AC mRNA and protein expression was repressed by IFN-γ in a concentration-dependent manner. IFN-γ showed limited effects on TGF-α and polyI:C-induced activation of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK). A chromatin immunoprecipitation assay indicated that Sp1 bound to its cognate sequence located on the *MUC5AC* promoter. The Sp1 inhibitor mithramycin A inhibited MUC5AC mRNA expression, implying a critical role for Sp1 in MUC5AC induction. Importantly, IFN-γ impeded Sp1 binding to the MUC5AC promoter.

Conclusions: These results suggest that IFN-γ represses MUC5AC expression, disturbing binding of Sp1 to its target sequences.

Key words:

MUC5AC, mucin, interferon-γ, bronchial epithelial cells, Sp1

Abbreviations

IFN-γ: interferon-γ
TGF-α: transforming growth factor-α
EGFR: epidermal growth factor receptor
ERK: extracellular signal-regulated kinase
IL-4: interleukin-4
IL-13: interleukin-13
NHBE: normal human bronchial epithelial
Th2: T helper 2
FBS: fetal bovine serum
ChIP: chromatin immunoprecipitation

Introduction

Mucus production by bronchial epithelial cells plays an important role in the clearance of pathogens from the lungs. However, excessive secretion of mucus in some disease states can generate mucus plugs, leading to impaired respiration and possibly, death (1, 2). Therefore, fine control of mucus secretion is important for the physiological functions of the airways.

MUC5AC, which is secreted mainly from goblet cells, is a major component of airway mucins (3). MUC5AC is upregulated in response to inflammation of the lungs caused by disorders such as bronchial asthma and airway infections (4). MUC5AC expression and secretion are induced by pro-inflammatory cytokines such as interleukin (IL)-4, IL-13, and transforming growth factor (TGF)-α (5), and by external agents such as viruses and cigarette smoke. In inflamed tissues, different stimuli can function simultaneously in the same locale while having diverse and interacting functions. Some costimuli initiate common effects, whereas others negatively regulate or synergize with each other. polyI:C, a mimic of viral double-stranded RNA, has been observed to enhance the effects of TGF-α, a ligand for the epidermal growth factor receptor (EGFR) (6). These two stimuli synergistically induce the production of MUC5AC. An underlying mechanism for this synergism is that polyI:C suppresses the expression of dual specificity phosphatase 6 (DUSP6), a negative regulator of extracellular signal-regulated kinase (ERK)1/2, which augments MAPK signaling pathways that are required for *MUC5AC* expression (6). As another example, CCL20 has also been shown to enhance EGFR-dependent MUC5AC production through its unique G protein-coupled receptor, CCR6 (7).

Interferon (IFN)- γ is a pro-inflammatory multi-functional cytokine produced by cells involved in Type 1 immunity, such as innate lymphoid cells, natural killer

cells, CD8+ cytotoxic T cells, and CD4+ T helper 1 (Th1) cells (8-10). IFN-γ activates macrophages, converting them to potent effector cells during infection of intracellular microbes, and it antagonizes the functions of T helper 2 (Th2) cell cytokines such as IL-4 and IL-13 (11). IFN-γ levels are usually decreased in individuals with bronchial asthma but increased in severe asthma (12). After infection with respiratory syncytial virus, mice deficient in the IFN-γ receptor show severe airway inflammation as indicated by damaged epithelial cells and excessive mucus production (13, 14). These findings suggest that IFN-γ plays a vital role in host defenses against viral infections and in the suppression of airway mucus production. However, little is known about the mechanisms by which IFN-γ suppresses mucin production.

We explored the roles of IFN-γ in mucin production, specifically *MUC5AC* transcription in human bronchial epithelial cells.

Materials and Methods

Cell culture and stimulation

The human pulmonary mucoepidermoid carcinoma cell line NCI-H292 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere with 5% (v/v) CO_2. Cells were grown until 80% confluence and kept in serum-free RPMI 1640 medium for 6 h before stimulation. Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Basel, Switzerland). NHBE cells were seeded at a density of $1.3 \times 10^5/cm^2$ into 12-well plates containing bronchial epithelial growth medium (Lonza) supplemented with defined growth factors and retinoic acid from the SingleQuot kit (Lonza), and were incubated at 37°C in a humidified atmosphere with 5% CO_2. The cells were exposed to 25 μg/ml polyI:C (Sigma-Aldrich, St. Louis, MO, USA) and 4 ng/ml TGF-α (R&D Systems, Minneapolis, MN, USA). IFN-γ (R&D Systems) was added at 30 ng/ml, unless otherwise indicated, simultaneously with polyI:C and TGF-α. Mithramycin A (Cayman chemical, Orland, FL, USA) was added 30 min prior to TGF-α and polyI:C stimulation.

Gene knockdown with short interfering RNA (siRNA)

siRNAs against Janus kinase 1 *(JAK1*) and signal transducers and activators of transcription-1 (*STAT1*) were used together with control scrambled siRNA (Life Technologies, Carlsbad, CA, USA). Two different siRNAs for each gene were used (s277 and s279 for *STAT1*, s7646 and s7647 for *JAK1*) to avoid off-target effects of RNA interference. NCI-H292 cells were seeded into 6-well plates and cultured to 50% confluence. The cells were transfected with 50 nM siRNA with 5 μl of Lipofectamine RNAiMAX (Life Technologies) and kept in RPMI with 10% FBS without antibiotics

for 24 h. Then, the medium was changed to serum-free RPMI 1640. After 12 h, the cells were treated with IFN-γ and polyI:C, and/or TGF-α for 12 h.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using the ToTally RNA kit (Life Technologies). Revers e transcription was performed with 1 μg of total RNA and oligo (dT) primers using SuperScript III (Life Technologies) according to the manufacturer's protoc ol. Relative mRNA levels were quantified with either SYBR Green or TaqMan gene expression assays (Life Technologies) on an ABI Prism 7900HT sequence detection system. Initial denaturation was performed at 95°C for 10 min, follo wed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extensio n at 60°C for 1 min. The threshold cycle time (CT) was recorded for each sa mple to reflect the level of mRNA expression. A validation experiment in a gi ven sample at different RNA concentrations confirmed linear dependence of th e CT value on the concentrations of the genes of interest and *GAPDH*, and th e consistency of ΔCT. ΔΔCT was used for relative mRNA quantitation. The fol lowing primers of TaqMan gene expression assays (Life Technologies) were us ed; *MUC5AC* (HS01365601_m1), *JAK1* (HS00233820_m1), *STAT1* (HS01014002_m 1), and *GAPDH* (4326317E). The following primers were used for SYBR Green assays; SP1, forward (5′-ACCAAGCTGAGCTCCATGAT-3′), and reverse (5′- CC TCAGTGCATTGGGTACTTC-3′); GAPDH (5′- GCACCGTCAAGGCTGAGAAC-3′) and reverse (5′- TGGTGAAGACGCCAGTGGA-3′).

Western blot analysis

Cells (3.0×10^5) were washed with PBS and lysed in 300 μl of lysis buffer [0.5% NP-40,

10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 3 mM *p*-amidinophenylmethanesulfonyl fluoride (Sigma-Aldrich), 5 mg/ml aprotinin (Sigma-Aldrich), 2 mM sodium orthovanadate (Sigma-Aldrich), and 5 mM EDTA]. Whole cell extracts were subjected to electrophoresis on 7.5–12% Tris-glycine gels (XVPantera Gel; DRC, Tokyo, Japan) and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T, pH 7.5) for 30 min at room temperature (RT) and probed with primary anti-human phosphor-p44/42 (Thr202/Tyr204) (D13.14.4E), p44/42 MAP kinase antibodies (137F5), anti-EGF Receptor (D38B1), anti-phospho-EGF Receptor (Tyr1068) (D7A5), anti-SP1 (D4C3) (all from Cell Signaling Technology Inc., Beverly, MA, USA), and anti-DUSP6 (ab76310; Abcam, Cambridge, UK) antibodies for 1 h at RT. Then, the membranes were washed with TBS-T and incubated with a secondary donkey anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (HRP) (GE Healthcare, Tokyo, Japan) for 1 h at RT. Proteins were detected using a chemiluminescence detection system according to the manufacturer's instructions (ECL Plus Western Blot Detection System; GE Healthcare).

Flow cytometry

Cells were collected using trypsin with 0.05% EDTA and fixed with 4% paraformaldehyde for 15 min. The cells were washed with FACS buffer [3% FBS, 0.05% NaN_3 in phosphate-buffered saline (PBS)] and blocked with blocking buffer (0.1% Triton-X 100, 3% bovine serum albumin in PBS) for 15 min. Then, the cells were incubated with mouse anti-MUC5AC antibody (45M1; Abcam) diluted at 1:200 in FACS buffer for 30 min. After washing with FACS buffer, the cells were incubated for 30 min with Alexa 488-conjugated anti-mouse IgG antibody (Life Technologies)

diluted at 1:400 in FACS buffer. The cells were washed with FACS buffer and subjected to flow cytometric analysis using a BD FACSAria II (BD Bioscience, Franklin Lakes, NJ, USA)

Enzyme-linked immunoassay (EIA)

MUC5AC protein was measured with EIA as described previously (6). Briefly, 50 µl of culture was collected at 12 h after stimulation and incubated overnight with bicarbonate-carbonate buffer (50 µl) at 40°C in a 96-well plate (Nunc Labware Products/Sigma-Aldrich). The plates were washed with PBS and blocked with 2% bovine serum albumin for 1 h at 37°C. Per well, 50 µl of mouse monoclonal anti-MUC5AC antibody (Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:100 with PBS containing 0.05% Tween-20 was added for 1 h. HRP-conjugated sheep anti-mouse IgG antibody (1:10,000) (GE Healthcare) was used as a secondary antibody. Color was developed with 3,3′,5,5′-tetramethylbenzidine peroxidase solution (KPL, Gaithersburg, MD, USA) and the reaction was stopped with 1 M H_2SO_4. The data were read by using Wallc ARVO SX (PerkinElmer, Waltham, MA, USA)

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as described previously (15). Cells were fixed wi th 1% formalin in PBS for 10 min at room temperature and incubated with 0. 125 M glycine for 5 min for quenching. The cells were collected and centrifug ed after washing three times with PBS. Pellets were suspended in swelling buf fer (25 mM Hepes, pH 8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM DT T, 1× protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), incubated on i ce for 10 min, and centrifuged. The pellets were homogenized with plastic ho

mogenizers. The cells were sonicated in sonication buffer (50 mM Hepes pH 8, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, protease inhibitor cocktail). Chromatin was sonicated with a Bioruptor (Cosmo Bio, Tokyo, Japan) and the soluble fraction of chromatin was incubated with mouse anti-SP1 antibody (SC-59; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. Antibody-conjugated chromatin was collected with 40 µL of Dynabeads conjugated with anti-mouse antibody (Life Technologies) by rotating for 1 h at 4°C. The beads were washed with low-salt (20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), high-salt (20 mM Tris-HCl pH 8, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), and LiCl (10 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% NP-40, 1% Na-deoxycholate) buffers for 5 min each, then washed twice with Tris-EDTA for 3 min. Immunoprecipitates were collected by incubation with elution buffer (1% SDS, 100 mM $NaHCO_3$) s for 15 min at 65˚C twice, treated with RNase and proteinase K, and purified. Coimmunoprecipitated DNA was detected by qPCR as described above using the following primers: CS-UP, forward (5′-CTGGCTGAGGGAGGAGAAA-3′) and reverse (5′-AGTGCCCCCTGCGTGTCT-3′); CS-DN, forward (5′-CCCACTGCCTCCTGTCTATC-3′) and reverse (5'- GCATAGGGAAGGGCTTGAG-3′); CS-Int, forward (5′-AGCAGCATAGCCCCTGACT-3′) and reverse (5′-CCCGAGATCTGAGCTCCTAC-3′); DUSP-UP, forward (5′- TCATGACGTTGGCATTTCTG-3′) and reverse (5′- GGGAAACAAGGTACCGTCAA-3′); and DUSP-Exn, forward (5′- CGTCCTTGAGCTTCTTGAGC-3′) and reverse (5′- ACAGTGGTGCTCTACGACGA-3′).

Statistical analysis

All data are expressed as the mean ± SD. Results were analyzed using one-way ANOVA followed by a post-hoc Tukey multiple comparison test (Figure 1A and B), post-hoc Dunnett test (Figure 6B), Mann-Whitney test (Figures 1E, 2) or Kruskal-Wallis test (Figure 5B). Analyses were performed with the GraphPad Prism software version 5.04 and $p<0.05$ was considered significant.

Results

IFN-γ decreases the expression of MUC5AC *mRNA*

Because IFN-γ plays a role in viral infections and allergic inflammation, and in both conditions mucus secretion from the airway is increased, we studied how IFN-γ influences mucin production from human bronchial epithelial cells. A combination of TGF-α and polyI:C (hereafter designated as "TP") was used based on our previous data showing that this combination robustly induces *MUC5AC* mRNA expression in NHBE and NCI-H292 cells (6). As expected, TP stimulation significantly increased *MUC5AC* mRNA expression (Figure 1A). Simultaneous addition of IFN-γ repressed the induction of *MUC5AC* in NHBE (Figure 1A). The inhibition by IFN-γ was also observed in NCI-H292 cells (Figure 1B). Immunocytochemistry for cytosolic MUC5AC proteins revealed that IFN-γ also reduced the TP-induced expression of the MUC5AC protein in the cytoplasm (Figure 1C). The number of cells expressing MUC5AC was markedly reduced by IFN-γ (Figure 1D). MUC5AC is released upon a variety of stimuli (5, 6, 16). The amount of MUC5AC in cellular secretion was determined by EIA. IFN-γ reduced the amount of secreted MUC5AC protein (Figure 1E). Although IFN-γ triggers apoptosis in some cell types (17), cells with condensed nuclei were not increased as judged by immunostaining and flow cytometric analysis (data not shown). Taken together, these results indicated that both mRNA and protein expression as well as release of MUC5AC can be repressed by IFN-γ.

The JAK/STAT pathway is essential for IFN-γ to decrease MUC5AC *mRNA expression*

IFN-γ binds to receptors on the cell surface to activate JAK1. Activated JAK1 phosphorylates STAT1, which then dimerizes, translocates to the nucleus, and binds

to its cognate sequence to induce target gene transcription (18). To determine whether JAK1 and STAT1 are involved in the IFN-γ-mediated suppression of TP-induced *MUC5AC* expression, knockdown experiments were performed by introducing JAK1 and STAT1 siRNA into cells. The siRNAs reduced the expression of both JAK1 and STAT1 (Supplemental Figure 1). Next, we stimulated these cells with TP in the presence or absence of IFN-γ. MUC5AC expression was significantly repressed in the presence of IFN-γ in control RNAi (Figure 2). The suppression was attenuated when either JAK1 or STAT1 were silenced, and more profoundly so when both were silenced simultaneously (Figure 2). This indicated that IFN-γ uses the JAK/STAT pathway to inhibit the transcription of *MUC5AC*.

IFN-γ partially inhibits the prolonged activation of EGFR and ERK1/2 induced by TGF-a and polyI:C

TGF-α binds to EGFR to transduce its signals into cells. Upon extracellular ligand binding, EGFR is dimerized and autophosphorylates multiple tyrosine residues positioned on its intracellular domain. EGFR was phosphorylated on tyrosine residue Y1068 by stimulation with TP in H292 cells (Figure 3A). The phosphorylation was detectable until 12 h after stimulation. It appeared to be slightly, albeit insignificantly, suppressed at later time points in the presence of IFN-γ as compared to in the absence thereof (Figure 3A).

A previous study demonstrated that the MAPK pathway is vital for the induction of *MUC5AC* expression by TGF-α (5). We also have shown that the MEK inhibitor U0126 strongly inhibits *MUC5AC* expression caused by stimulation with TP (6), which prompted us to study how IFN-γ influences the activation of the MAPK signaling pathway. ERK1/2 was activated by TP stimulation. The phosphorylation

gradually decreased, but was still significantly elevated even 12 h after stimulation (Figure 3B). Simultaneous supplementation with IFN-γ did not reduce ERK phosphorylation from 3 to 12 h (Figure 3B). Several negative regulators of the MAPK pathways (19), including DUSP6, an ERK-specific phosphatase, are known. We have previously shown that reduction of DUSP6 expression by polyI:C underlies the synergistic induction of *MUC5AC* expression by stimulation with TP. We evaluated whether DUSP6 is involved in the reduction of phosphorylation of ERK observed at later time points. Western blot analysis showed that stimulation with TP markedly reduced DUSP6 protein expression as compared to the control (Figure 3C), which is consistent with our previous observations (6). Contrary to our expectation, however, IFN-γ did not ameliorate the reduced DUSP6 expression (Figure 3C). These results indicated that IFN-γ did not strongly inhibit activation of either EGFR or EKR, or induce DUSP6 expression, suggesting that IFN-γ represses MUC5AC expression through other mechanisms.

Reduced binding of Sp1 to the* MUC5AC *promoter is involved in the inhibitory effects of IFN-γ

MUC5AC is induced in human bronchial epithelial cells by a wide variety of stimuli including cigarette smoke and viral infection, in addition to EGFR stimulation by cytokines. Several transcription factors such as NF-κB and Sp1 play pivotal roles in *MUC5AC* expression in response to such stimuli. We have previously shown that NF-κB plays a limited role in TP-induced expression of *MUC5AC* (6, 20). Therefore, we aimed to determine whether Sp1 participates in the transcriptional activation of *MUC5AC* by TP. Mithramycin A, a specific inhibitor of Sp1 binding to DNA, inhibited TP-induced *MUC5AC* expression in a concentration-dependent manner (Figure 4),

which indicates that Sp1 plays a crucial role in *MUC5AC* expression in response to stimulation with TP.

There are two consensus Sp1 binding sites located at the 3' upstream region of the transcription start site of *MUC5AC* (Figure 5A). One of these binding sites was previously shown to be of paramount importance in cigarette smoke-induced expression of *MUC5AC* by a promoter assay (21). Hence, we investigated which consensus binding sites are occupied by Sp1 after stimulation with TP. The ChIP assay revealed that Sp1 binds to a consensus binding site located ~3.5 kb upstream of the transcriptional start site of *MUC5AC* (CS-DN), but does not bind to the ~3.7-kb upstream site (CS-UP) (Figure 5A and B). A consensus Sp1 binding site within the first intron (CS-Int) and negative control sequences of the promoter (DUSP-UP) and the first exon (DUSP-Exn) of *DUSP6* were not bound by Sp1. Furthermore, binding of Sp1 to CS-DN was decreased to control levels by treatment with IFN-γ. Because in a cigarette smoke model, an increase in SP1 binding to the *MUC5AC* promoter was dependent on an increase in SP1 synthesis (21), we checked the expression level of SP1 after IFN-γ stimulation. *SP1* mRNA expression 3 h after TP stimulation, which was the same period as that used for the ChIP experiment, was not affected by the presence of IFN-γ (Figure 5C). SP1 protein increased in a time-dependent manner after TP or TPI stimulation and appeared to decrease 12 h later in the presence of IFN-γ (Figure 5D). However, 3 h after stimulation, the protein amount was hardly affected by IFN-γ (Figure 5E). This indicates that a decrease in SP1 binding to the MUC5AC promoter observed with ChIP assay (Figure 5B) is not due to a reduction of SP1 expression. Taken together, these results demonstrated specific binding of Sp1 to the promoter of *MUC5AC* and the inhibition thereof by IFN-γ, and suggest that IFN-γ inhibits *MUC5AC* transcription at least in part by impeding Sp1 from binding to one of its

cognate sequences on the *MUC5AC* promoter.

Discussion

Mucus secretion is one of the most important defense systems to eliminate pathogens or allergens from the airway epithelium (22, 23). Roy et al. (22) showed that a lack of MUC5AC had little effect on the clearance of bacteria from the airways, whereas a lack of MUC5B delayed pathogen clearance. However, overexpression of MUC5AC in the airways increased the clearance of pathogens (24), indicating that secretion of this mucin into the airways protects against infection. In contrast, mucin hypersecretion from bronchial epithelial cells can cause airflow obstruction in inflammatory airway diseases such as bronchial asthma, and in bacterial or viral infections. Therefore, fine control of mucin production, or a balance between positive and negative regulation of mucin expression, is of great importance in maintaining airway homeostasis.

IFN-γ is a pro-inflammatory, multifunctional cytokine that is produced by Type 1 innate lymphoid cells, natural killer cells, CD8+ cytotoxic T cells, and Th1 cells (8-10). IFN-γ suppressed mucus production in a murine airway allergy model (25). Transplantation of allergen-sensitized Th1 cells into airway allergy model mice suppressed eosinophil infiltration, mucus production in the airways, and production of antigen-specific IgE (25). Furthermore, in mice lacking the IFN-γ receptor, some of the effects achieved by transplantation of antigen-sensitized Th1 cells, including mucus production, disappeared. In the current study, we demonstrated that IFN-γ suppresses mucin production in human airway epithelium cells by inhibiting transcription of the *MUC5AC* gene, which was induced by other stimuli.

EGFR was activated by prolonged (≥12 h) phosphorylation of Y1068 in

response to TGF-α and polyI:C (Figure 3A). Such prolonged phosphorylation was slightly repressed by IFN-γ (Figure 3A). IFN-γ has diverse influences on EGFR activation. For example, Burova et al. (26) reported that IFN-γ induced transactivation of EGFR in A431 and HeLa cells. In contrast, IFN-γ was shown to inhibit EGFR activation, specifically phosphorylation of Y1068, in colonocytes (27). Furthermore, in a melanoma cell line, IFN-γ reduced the cell surface expression of EGFR protein (28). These variable responses might be explained by differences in the cell types used, the differentiation status of the cells, or the experimental conditions. The mechanisms underlying these diverse functions of IFN-γ remain to be elucidated.

We observed that IFN-γ did not suppress phosphorylation of the MAPK signaling molecules ERK1/2 at later time points. ERK1/2 is activated by IFN-γ in macrophages (23) and inhibited in thyroid cells (29). The different effects of IFN-γ between other studies and ours likely reflect the pleiotropic and context-dependent nature of IFN-γ functions.

In addition to EGFR and MAPK, we focused on the transcription factor SP1, which has been implicated in *MUC5AC* gene expression induced by a wide variety of stimuli in bronchial epithelial cells (21). TP-induced Sp1 binding to the *MUC5AC* promoter was almost abolished by IFN-γ. We concluded that inhibition of Sp1 binding to the *MUC5AC* promoter by IFN-γ participates in the inhibition of *MUC5AC* expression.

IFN-γ is a well-conserved and widely distributed cytokine that plays a central role in cellular immunity (11). The most prominent and well-characterized function of IFN-γ released from Th1 cells is to suppress inflammatory responses mediated by Th2 cell-derived cytokines such as IL-4 and IL-13. These activities of IFN-γ are mediated primarily through the activation of JAK kinases and STAT, a latent transcription factor,

by inducing expression of genes through the binding of STATs to gamma activating sequences on their promoters. However, a previous study showed that IFN-γ can also repress gene expression (30) through as-yet unknown mechanisms. Intriguingly, Trilling et al. (30) performed a microarray-based analysis of genes that were up- or downregulated without protein synthesis by IFN-γ in NIH-3T3 fibroblasts, and found that genes downregulated by IFN-γ showed significant enrichment of GC-box and Sp1 binding sequences in their regulatory regions. This observation is consistent with our current finding that IFN-γ inhibits the expression of *MUC5AC* through the release of Sp1 from the *MUC5AC* promoter. Because we used different cells than those used by Trilling et al., the similar results suggest that the suppression of Sp1-induced gene expression by IFN-γ is a general rather than a cell-type-specific phenomenon.

In conclusion, the mechanisms underlying *MUC5AC* transcriptional inhibition by IFN-γ though the release of Sp1 remains largely unknown. Nevertheless, we conclude that IFN-γ is a negative regulator of MUC5AC production from human bronchial epithelial cells at the transcriptional level.

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Figure legends

Figure 1. IFN-γ decreases the expression of MUC5AC. A. MUC5AC mRNA expression in NHBE cells. qRT-PCR was performed for *MUC5AC* mRNA with NHBE cells treated with or without 30 ng/mL of IFN-γ at the indicated concentrations in the presence of TGF-α or polyI:C, or both. Data were normalized to the control (without any stimulation). *p<0.05 one-way ANOVA with Tukey's post-test (n=6). B. *MUC5AC* mRNA expression in NCI-H292 cells. qRT-PCR was performed for *MUC5AC* mRNA with H292 cells treated with IFN-γ at the indicated concentrations in the presence of TGF-α and polyI:C. Data were normalized to the control (without any stimulation). ***p<0.001 one-way ANOVA with Tukey's post-test (n=7). C. Representative images of immunostaining for cytoplasmic MUC5AC in H292 cells stimulated with TGF-α and polyI:C with (TPI) or without IFN-γ (TP) for 12h. D. Flow-cytometric analysis of *MUC5AC* expression. Cells were stimulated with TP or TPI for 12 h, fixed, and stained with an anti-MUC5AC antibody and then with FITC-conjugated anti-mouse IgG secondary antibody. The numbers of MUC5AC-positive cells were determined by flow cytometry. E. Quantitation of MUC5AC protein secreted into the culture media. The amount of MUC5AC was determined with enzyme-linked immunoassay (EIA). Data were normalized to the control (without any stimulation). *p<0.05 with Mann-Whitney test. n=6.

Figure 2. Effects of RNAi for JAK1 and/or STAT1 on the expression of *MUC5AC*. Cells were transfected with siJAK1 and/or siSTAT1 and stimulated with TGF-α and polyI:C with or without IFN-γ for 12 h in the presence or absence of IFN-γ. *MUC5AC* mRNA expression was determined by qRT-PCR and was normalized to *GAPDH* mRNA. #1

and #2 indicate different combinations of siRNA probes for STAT1 and JAK1; #1 for IDs: s277 and s7646, #2 for s279 and s7647, respectively. The significant suppression of *MUC5AC* mRNA by IFN-γ was most significantly prevented by treatment with a combination of siJAK1 and si-STAT1. Data were analyzed with a Mann-Whitney test (n=6–12).

Figure 3. Effects of IFN-γ on EGFR and ERK phosphorylation. Cells were stimulated with TGF-α and polyI:C with (TPI) or without IFN-γ (TP) for the indicated times and subjected to western blotting. Representative results are shown. A. Phosphorylation of EGFR at Y1068 after stimulation for 1–12 h. Whole cell lysates were subjected to western blotting using anti-EGFR and anti-phospho-EGFR (Y1068) antibodies. Band intensities of phospho-EGFR (pEGFR) and EGFR in the repeated experiments (n=7) were quantified. B. Phosphorylation of ERK 1/2 after stimulation for 1–12 h. Anti-ERKs and anti-phospho-EKRs (pERK) antibodies were used. Band intensities of pEGFR and EGFR in the repeated experiments (n=7) were quantified. C. DUSP6 protein expression. Cells were stimulated with TGF-α and polyI:C with (TPI) or without IFN-γ (TP) for the indicated times and subjected to western blotting. Anti-DUSP6 antibody was used.

Figure 4. The role of SP1 in IFN-γ-mediated repression of *MUC5AC* expression. A. Effect of mithramycin A, an SP1 inhibitor, on TGF-α- and polyI:C-induced *MUC5AC* mRNA expression. Cells were treated with mithramycin A for 30 min before stimulation at the indicated concentrations. *MUC5AC* expression was determined with qRT-PCR (n=3).

Figure 5. Sp1 binding to *MUC5AC* promoter was inhibited by IFN-γ. A. Schematic representation of the *MUC5AC* gene. Consensus binding sites for SP1 at -3.7 (CS-UP) and -3.5 kb (CS-DN) upstream of the transcription start site and at the first intron (CS-Int) are indicated. B. ChIP for SP1 at the site indicated in A and negative control sequences on the promoter (DUSP-UP) and exon 1 (DUSP-Exn) sites of *DUSP6*. $^{**}p<0.01$; Kruskal-Wallis test (n=9). C. qRT-PCR analysis of SP1 expression after TGF-α and polyI:C stimulation with (TPI) or without IFN-γ (TP) (n=6). D. Western blot for SP1 expression with TP or TPI stimulation for the indicated periods. E. Quantitation of blot intensities at 3 h after TP or TPI stimulation (n=5).

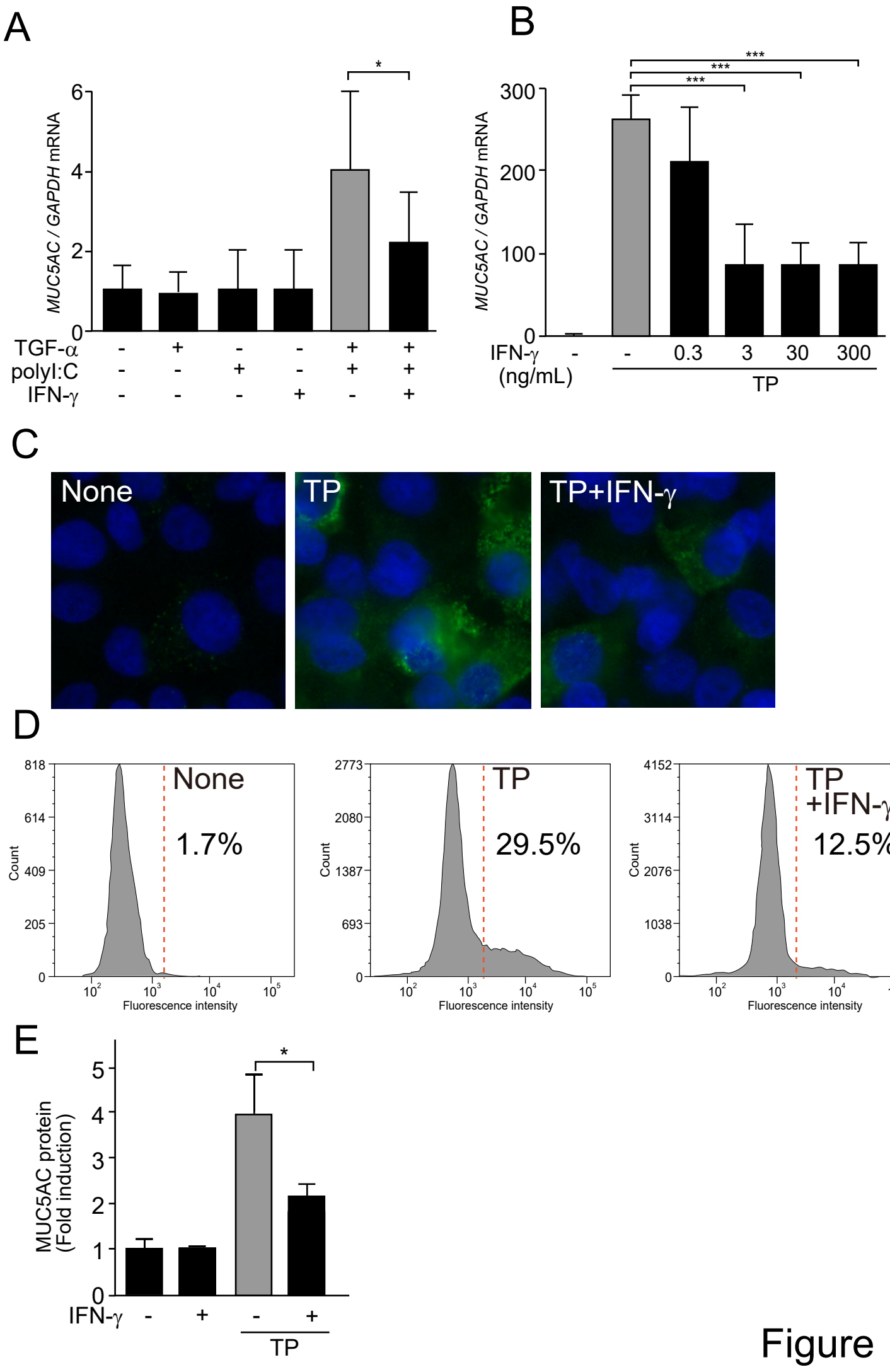

A
MUC5AC / GAPDH mRNA
TGF-α
polyI:C
IFN-γ
B
MUC5AC / GAPDH mRNA
IFN-γ (ng/mL)
TP
C
None
TP
TP+IFN-γ
D
None
1.7%
TP
29.5%
TP +IFN-γ
12.5%
Count
Fluorescence intensity
E
MUC5AC protein (Fold induction)
IFN-γ
TP

Figure 1

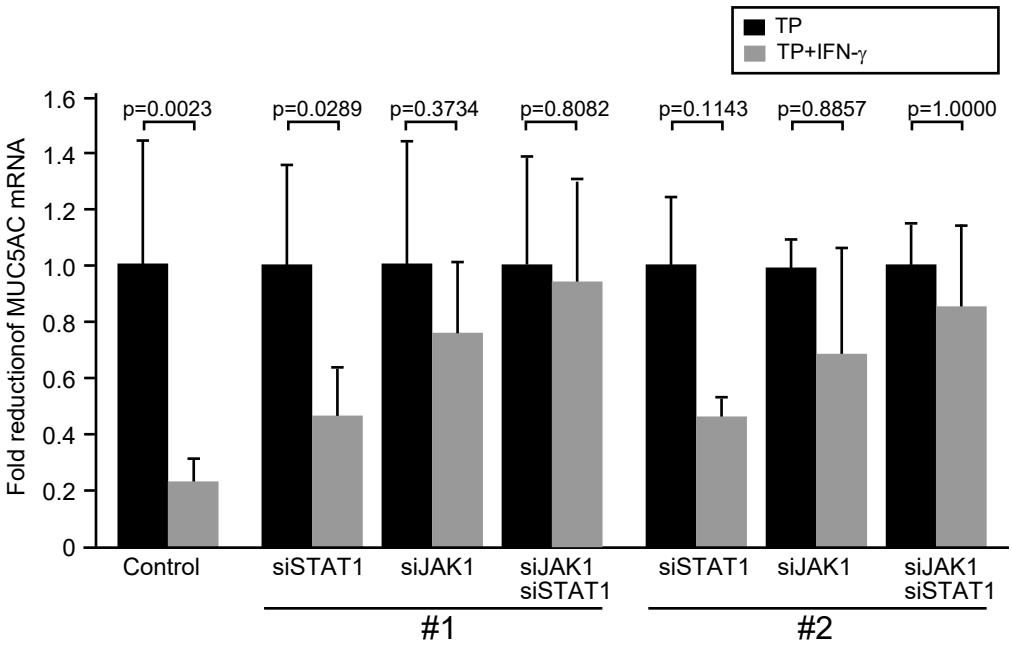

TP
TP+IFN-γ
Fold reductionof MUC5AC mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
p=0.0023
p=0.0289
p=0.3734
p=0.8082
p=0.1143
p=0.8857
p=1.0000
Control
siSTAT1
siJAK1
siJAK1
siSTAT1
siSTAT1
siJAK1
siJAK1
siSTAT1
#1
#2

Figure 2

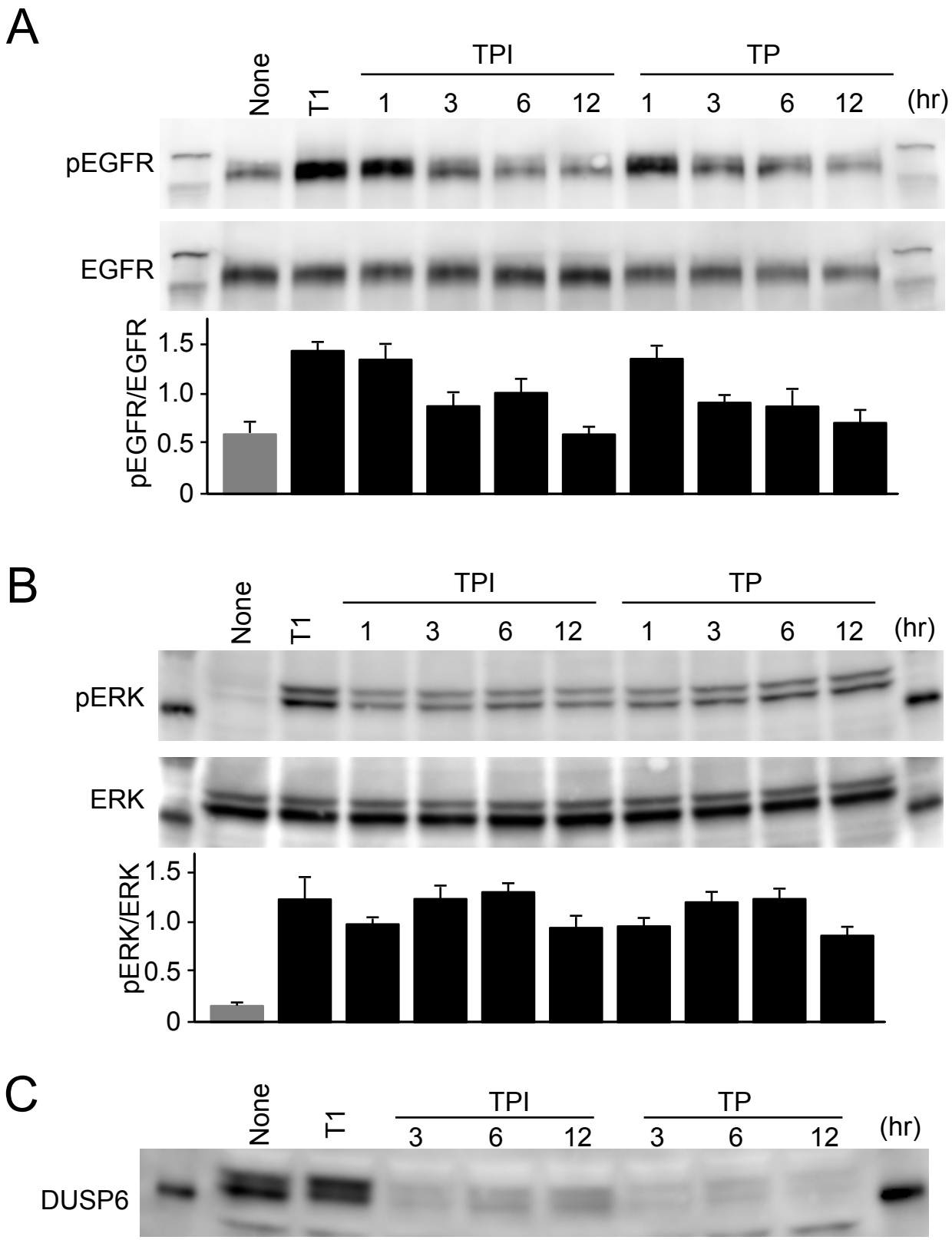

A
None
T1
TPI
TP
1
3
6
12
1
3
6
12
(hr)
pEGFR
EGFR
pEGFR/EGFR
1.5
1.0
0.5
0
B
None
T1
TPI
TP
1
3
6
12
1
3
6
12
(hr)
pERK
ERK
pERK/ERK
1.5
1.0
0.5
0
C
None
T1
TPI
TP
3
6
12
3
6
12
(hr)
DUSP6

Figure 3

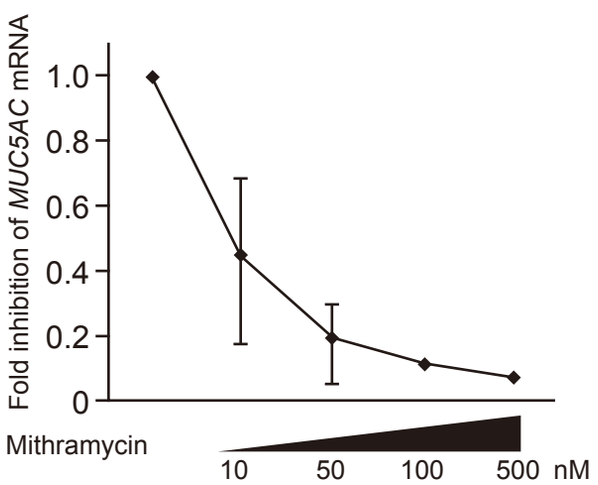

Fold inhibition of MUC5AC mRNA
1.0
0.8
0.6
0.4
0.2
0
Mithramycin
10
50
100
500 nM

Figure 4

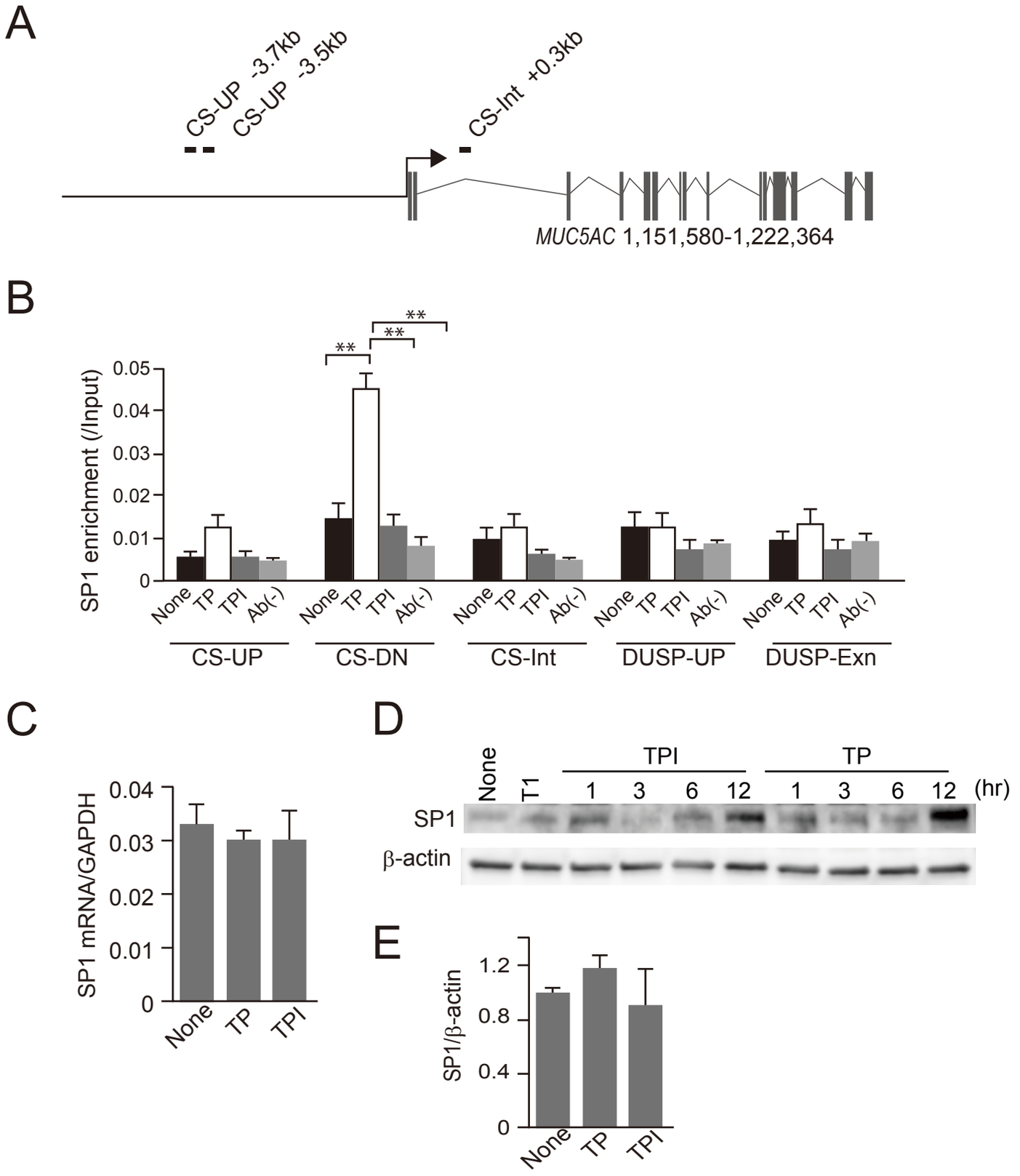
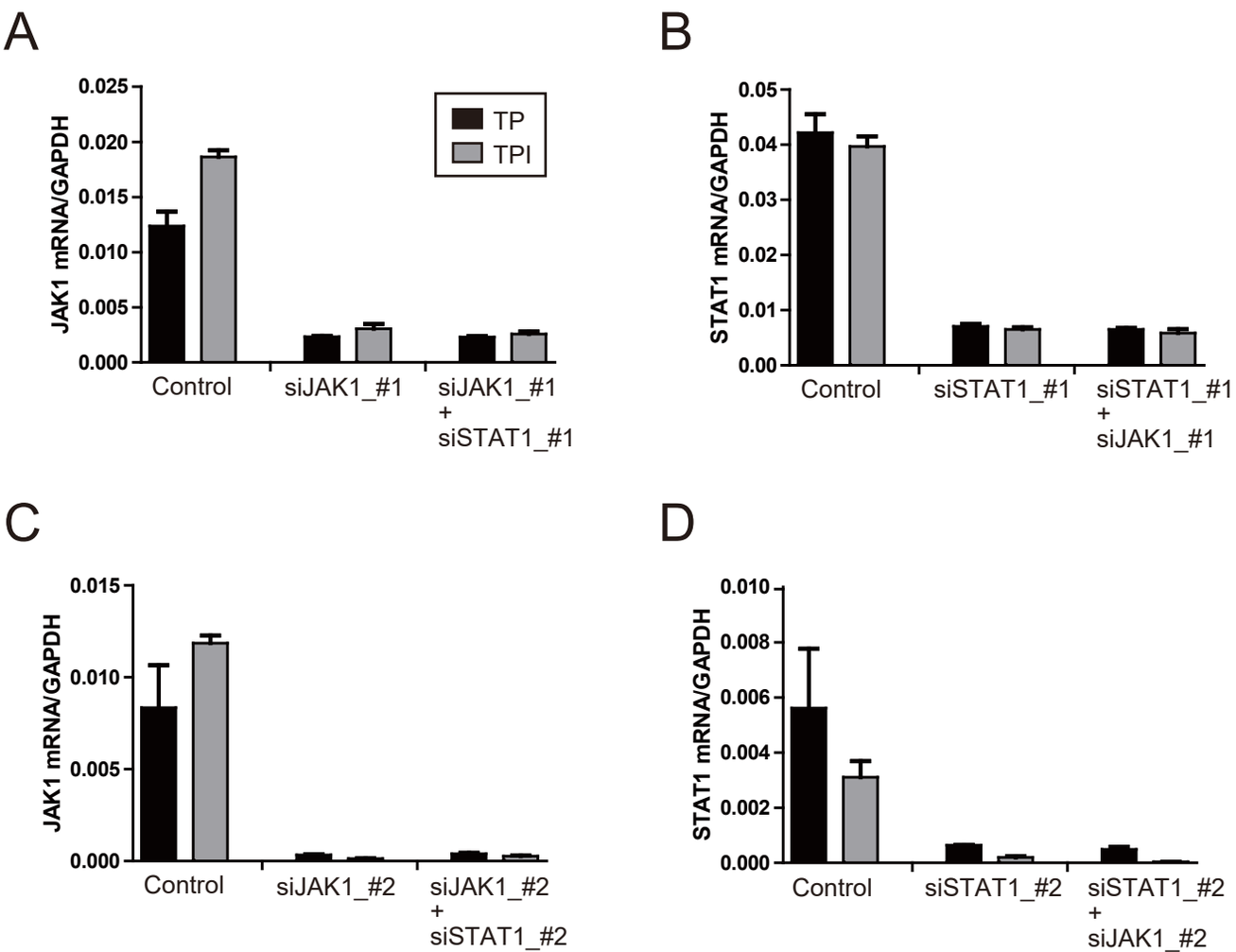

A
CS-UP -3.7kb
CS-UP -3.5kb
CS-Int +0.3kb
MUC5AC 1,151,580-1,222,364
B
SP1 enrichment (/Input)
0.05
0.04
0.03
0.02
0.01
0
**
**
**
None
TP
TPI
Ab(-)
CS-UP
CS-DN
CS-Int
DUSP-UP
DUSP-Exn
C
SP1 mRNA/GAPDH
0.04
0.03
0.02
0.01
0
None
TP
TPI
D
None
T1
TPI
TP
1
3
6
12
(hr)
SP1
β-actin
E
SP1/β-actin
1.2
0.8
0.4
0
None
TP
TPI

Figure 5



Supplementary Figure 1. Effects of siRNA on expression of target genes. Knocking down of JAK1 (A and C) and STAT1 (B and D) by siRNA probes for STAT1 and JAK1; #1 for IDs: s277 and s7646 (A and B) , #2 (C and D) for s279 and s7647 (A and B) were verified by qRT-PCR.